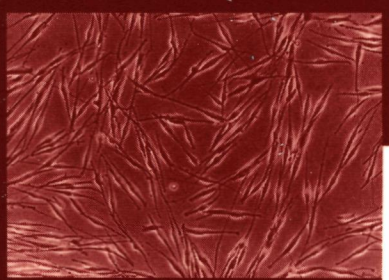
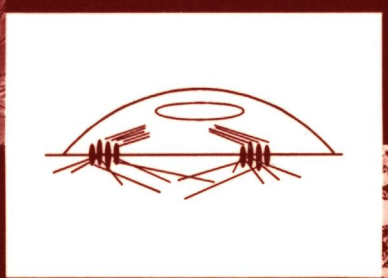
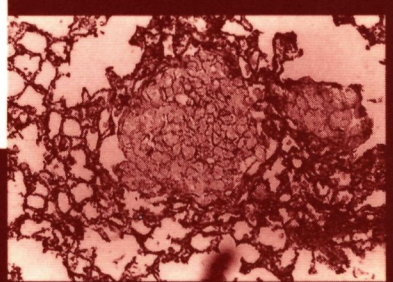
Cell adhesion receptors in human melanoma







Erik HJ Danen

Cell adhesion receptors in human melanoma

Cell adhesion receptors in human melanoma

Een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag, 28 maart 1996,
des namiddags om 1.30 uur precies

door

Erik Hendrik Julius Danen
geboren 11 april 1965 te Dieren.

Promotor: Prof. Dr. D.J. Ruiter
Co-promotor: Dr. G.N.P. van Muijen

The research presented in this thesis was performed in the Department of Pathology, University Hospital, Nijmegen, The Netherlands. The studies were financially supported by grant NUKC 91-09 from the Dutch Cancer Society and by the European Community Concerted Action on melanoma progression.

Manuscriptcomissie: Prof. Dr. C.G. Figdor
Dr. E. Roos
Prof. Dr. B. Wieringa

Offset: Ponsen & Looijen b.v.

Financial support for the printing of this thesis was provided by the Dutch Cancer Society.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Danen, Erik Hendrik Julius

Cell adhesion receptors in human melanoma / Erik Hendrik Julius Danen. - [S.l. : s.n.]. - Ill.
Proefschrift Katholieke Universiteit Nijmegen. - Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-9009194-7
Trefw.: kankeronderzoek

..... Also in the water of flower vases there was movement....: tapered, agile, crooked like old slippers, they flashed by at such speed that in order to follow them the magnification had to be reduced: they navigated in the ocean of a drop of water, rotating on their axis, smashing into obstacles, and then immediately turned around and were off again like crazed speedboats. They seemed to be hunting for light and air, solitary and bustling:

but I saw two of them put on the brakes as if one had noticed the other, as if they had taken a liking to each other; I saw them get close, adhere tightly, and continue the voyage together at a slower pace, as if by these blind nuptials they had exchanged something and from it drew a mysterious, infinitesimal pleasure.

Primo Levi, Other people's trades: "The invisible world".

Contents

Abbreviations

aPM	advanced primary melanoma
BSA	bovine serum albumin
CCBD	central cell binding domain
CD44s	standard CD44 isoform lacking all variant exon products
CD44v5	CD44 isoform containing variant exon 5 product
Co	collagen
CS(PG)	chondroitinsulphate (proteoglycan)
DMEM	Dulbecco's modified Eagle's medium
DN	dysplastic nevus
ECM	extracellular matrix
ePM	early primary melanoma
FCS	fetal calf serum
Fg	fibrinogen
FITC	fluorescein-isothyocianate
Fn	fibronectin
3Fn9	9th type III fibronectin repeat
h	hour
HA	hyaluronate
HS(PG)	heparansulphate (proteoglycan)
HUVEC	human umbillical vein endothelial cell
Ig	immunoglobulin
ICAM	intercellular adhesion molecule
i.v.	intravenous
Ln	laminin
mAb	monoclonal antibody
MCT	melanocyte
min	minute
MM	melanoma metastasis
NN	common nevocellular nevus
PBS	phosphate-buffered saline
PM	primary melanoma
PMA	phorbol 12-myristate 13-acetate
s.c.	subcutaneous
s.d.	standard deviation
TNFα	tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule
VLA	very late antigen
Vn	vitronectin

Chapter 1

General introduction

Parts of this chapter are published in *Cancer Surveys* **24**, 43-65, 1995

The process of tumor progression involves a series of sequential steps leading to metastasis. For several of these steps, tumor cells must be equipped with the appropriate adhesive phenotype. The molecules that mediate cell adhesion can be grouped in several distinct families; selectins, members of the immunoglobulin superfamily, cadherins, integrins, and CD44 molecules. In this chapter, the general properties of each of these families are introduced and their involvement in cancer, specifically in melanoma, is discussed. Since direct evidence for a role for adhesion molecules in melanoma growth and metastasis in vivo is limited to the integrins and CD44, these families are discussed in more detail.

In the establishment of a primary tumor, genetic changes occur, including activation of proto-oncogenes and inactivation of tumor suppressor genes, that lead to cellular transformation [14]. During these initial steps in oncogenesis, cells become less differentiated and their growth is no longer contact inhibited and often growth factor independent. Further tumor progression involves a series of sequential steps eventually resulting in the outgrowth of distant metastases [46]. It is this ability to metastasize that contributes most to the lethality of the disease.

This thesis focusses on melanoma, a cancer originating from melanocytes, the pigment producing cells in the skin (cutaneous melanoma) or in the eye (uveal melanoma). Cutaneous melanoma is an ideal model to study tumor progression because lesions of the different stages that are defined clinically, histopathologically, and immunologically are attainable [29,64], and because many well characterized human melanoma cell lines are available.

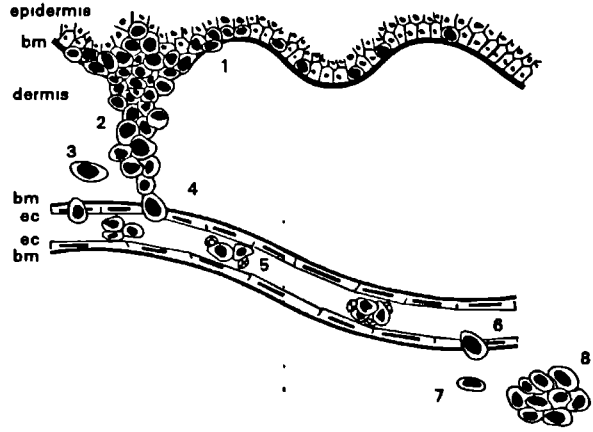


Figure 1: *Adhesive events in melanocytic tumor progression. Tumor cells penetrate the epidermal basement membrane (bm) (1) and invade into the dermis (2). They detach from the primary tumor, migrate through the dermis (3) and penetrate the basement membrane and endothelial cell layer (ec) of a blood vessel (intravasation) (4). In the blood stream, tumor cells may adhere to platelets (5). Finally, they extravasate at a distant site (6), followed by invasion (7) and proliferation (8) in the target organ.*

In order to be able to move through the steps of tumor progression and metastasize, tumor cells must acquire a number of molecular properties, many of which effect their

adhesive behavior (Fig 1) [207]. Tumor cells have been demonstrated to produce qualitatively or quantitatively altered extracellular matrix (ECM) deposits [134], to have reduced contacts with neighboring cells [191], and to synthesize elevated levels of proteases [118]. These features are likely to be important for the detachment from the primary tumor mass and for invasion into the surrounding ECM. Stimulation of angiogenesis [206] and expression of cellular adhesion molecules recognizing basement membrane components [194], may enable tumor cells to enter the blood stream. Downmodulation of expression of major histocompatibility antigens [119] and binding to platelets [84], can help tumor cells to circumvent the immune surveillance. The ability to adhere to endothelial cells [142] may be important for extravasation, and finally, mimicking the adhesive phenotype of activated lymphocytes may permit them to retain and proliferate in lymph nodes [65].

Thus, invasive and metastatic behavior requires various adhesive interactions of tumor cells with other cells and with ECM components. To date, several cell adhesion receptor families have been defined, including selectins, members of the immunoglobulin (Ig) superfamily, cadherins, integrins, and CD44 molecules (Fig 2) [74,102].

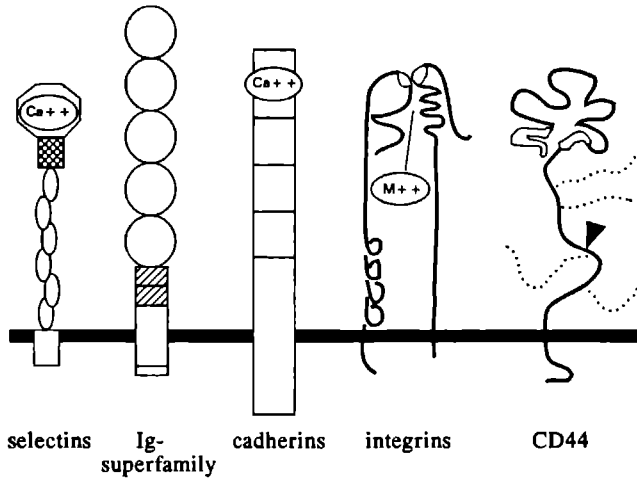


Figure 2. *Cell adhesion receptor families. For selectins, Ig superfamily members, and cadherins, the arrangement of known repeats is shown. For integrins and CD44, a schematic arrangement of the polypeptide chains is drawn. The filled horizontal bar indicates the cell membrane. Ligand binding domains are represented by dotted areas. The octagon depicts the lectin domain, the double hatched bar depicts the EGF-like repeat, ovals represent complement-binding protein related repeats, hatched bars represent fibronectin type III repeats, and circles represent Ig repeats. Ca^{2+}-dependent domains and metal (m) binding sites are indicated. For CD44, dotted lines are chondroitin sulphate chains and the triangle points the position where variant exons can be inserted. For references see text.*

Selectins

Three members of the selectin family have been identified, termed E-, P-, and L-selectin, and they are also referred to as LECAMs [11,96,193]. Each is a transmembrane molecule and has a Ca^{2+}-dependent C-type lectin domain, an EGF-like repeat, and various repeats with similarity to complement-binding proteins. They heterophilically (contact between different molecules) bind specific carbohydrate moieties via their lectin domains [146]. P- and L-selectin ligands are mucin-like molecules such as CD34, GlyCAM, and the mucosal addressin MAdCAM, all expressed on endothelial cells [193]. The ligand for E-selectin (ESL-1) was recently cloned and shown to be 94% identical to the fibroblast growth factor receptor [179].

Selectins are expressed on leukocytes and endothelial cells and mediate adhesion between these cell types. This interaction slows the leukocytes down (rolling), probably allowing factors in the local environment to activate them. Subsequently, firm adhesion is induced (involving integrins and members of the Ig superfamily), followed by lymphocyte homing into lymph nodes and leukocyte extravasation at sites of inflammation [170,175]. Studies with P-, L-, or E-selectin-deficient mice, combined with antibody inhibition studies, clearly demonstrate the crucial role for selectins in these processes [*see* 193].

Ig superfamily members

The Ig superfamily comprises a large number of adhesion molecules, all typified by several Ig-related domains and, usually, by several fibronectin type III repeats [209]. Most members are transmembrane molecules but phosphatidylinositol anchorage has also been found. N-CAM was the first member to be identified [30] and it belongs to a group that mediates homophilic (contact between identical molecules) cell adhesion. The members of a second group, i.e. ICAM-1, -2, and -3, VCAM-1, and MAdCAM-1 bind to members of the integrin family [10,16,36,41,147,178].

The ICAMs play a pivotal role in leukocyte interactions in various steps of an immune response and in the multistep cascade of leukocyte extravasation [173]. Furthermore, recent findings show that ICAM-1 [148] and ICAM-3 [80] can act as mediators of signal transduction as well as cell adhesion.

Cadherins

Members of the cadherin family are transmembrane molecules that mediate adhesion between cells in a homophilic, Ca^{2+}-dependent manner [55,74,188-191]. In addition, E-cadherin on keratinocytes can heterophilically bind integrin $\alpha e\beta 7$ on lymphocytes [23]. Extracellularly, cadherins consist of four homologous repeats, and the ligand binding region is located in the most distal repeat [129,166]. Intracellularly, they are linked to the cytoskeleton via a complex of intermediate proteins (catenins for E-cadherin), and these

associated molecules are essential for the adhesive function [86,123,133]. A large number of different cadherins are known, that all have their own specific pattern of spatial and temporal regulation during embryonic development, and their own pattern of tissue distribution [55,90,183,188-190].

Selective cadherin-mediated adhesion of cells to cells of their own type, is thought to be fundamental in the control of development and maintenance of tissues [128,188-190]. In adult normal tissue, specific cadherins are usually clustered in specialized regions of contact between the plasma membranes of opposing cells. For example, E-cadherin molecules are concentrated in adherens junctions of epithelial cells, whereas in desmosomal junctions of several different cell types, desmogleins and desmocollins are clustered [15,48,98,161,198].

Integrins

Structural aspects: Integrins form a family of heterodimeric transmembrane receptors [73]. Their α- and β-subunits are derived from separate genes and form glycoproteins with a generally conserved structure, made up of a large extracellular domain (ca. 1000 aminoacids for α-subunits and 750 aminoacids for β-subunits), a transmembrane segment, and a very short cytoplasmic tail (with the exception of the β4-subunit, *see below*). Complete sequence information has been obtained for most α- and β-chains [*see databanks and references in* 37,73,107,155]. The high conservation of β1 cytoplasmic domains across vertebrates, invertebrates, and even fungi, indicates the early origin of the integrins in evolution [111]. The subunits non-covalently associate in the cytoplasm and are subsequently transported to the cell surface [27]. Divalent cations are required for dimerization [53] and involvement of the chaperone calnexin has been reported [99].

Diversity within the family of integrins is generated by a large number of α-subunits which form heterodimers with at least nine different β-subunits (Table 1). The fact that one α-subunit can associate with more than one β-subunit, and the existence of alternative splicing for α- and β-chains further enhances the complexity of the family.

Ligand binding: Extracellularly, integrins can bind ECM components or counter receptors on other cells in a divalent cation-dependent way (Ca^{2+}, Mg^{2+}, or Mn^{2+}). Redundancy is a common feature in the integrin family as several integrins bind the same ligand and one integrin may bind several ligands (Table 1). Chemical cross-linking studies and mutational analyses have narrowed down the ligand binding site to a small region in the N-terminal domains of both subunits [*see* 73,107]. A highly conserved sequence in this region in the β-subunits, has homology to the calcium-binding loop of EF-hand proteins, and several lines of evidence suggest that the ligand interacts with divalent cations bound to this site [39,107]. A number of integrin recognition sites have been molecularly defined in various ligands and the best studied of those is the RGD sequence. It is present within several ECM proteins, and it is recognized by a large number of integrins (Table 1) [153].

Table 1. The integrin family.

subunits		*ligands*	*recognition sites*
	α1	Ln,Co	
	α2	Ln,Co,Tn,α3β1	DGEA
	α3*	Ln,Co,Fn,Ep,En,α2β1,α3β1	
	α4	Fn,VCAM1	EILDV,IDAPS,REDV
β1*	α5	Fn	RGD(+PHSRN)
	α6*	Ln	
	α7*	Ln	
	α8	Tn	
	αv	Fn,Vn	RGD
	α9	Ln,Co,Tn	
	αl	ICAM1,ICAM2,ICAM3	KELLLPGNNRKV
β2	αm	iC3B,Fb,FX,ICAM1	KQAGDV
	αx	iC3B,Fb	GPRP
β3*	αIIb	Fb,Fn,VWF,Vn,Ts	RGD(+PHSRN),KQAGDV
	αv	Vn,Fb,Fn,VWF,Ts,Os,Co,Tn	RGD(not PHSRN)
β4	α6*	Ln	
β5	αv	Vn	RGD
β6	αv	Fn	RGD
β7	α4	Fn,VCAM1,MAdCAM	EILDV
	αe	E-cadherin	
β8	αv	Vn	RGD
β9	α?	?	

*Alternative splicing reported. Abbreviations used: Ln=laminin, Co=collagen, Tn=tenascin, Fn=fibronectin, Ep=epiligrin, En=entactin, VCAM=vascular cell adhesion molecule, Vn=vitronectin, ICAM=intercellular adhesion molecule, iC3b-inactivated complement component C3, Fb=fibrinogen, Fx=factor X, VWF=von willebrand factor, Ts=thrombospondin, Os=osteopontin, MAdCAM=mucosal addressin cell adhesion molecule. For references see text.

Integrins of the β1,2,3 subfamilies have been most extensively studied. The β1- and β3-integrins are mainly involved in cell-ECM interactions whereas β2-integrins mediate cell-cell adhesion. However, β1-integrins can also be involved in cell-cell adhesion. E.g.,

$\alpha 4\beta 1$ is the receptor for vascular cell adhesion molecule (VCAM)-I [41]; $\alpha 3\beta 1$ can be localized at cell-cell contact sites [85]; $\alpha 2$ and $\alpha 3$ subunits accumulate in areas of cell-cell contacts in cultured keratinocytes [95]; binding of affinity purified $\alpha 2\beta 1$ to $\alpha 3\beta 1$ has been demonstrated [186]; and finally, homophylic $\alpha 3\beta 1$ interactions have been reported [181].

The finding that the binding specificities of a certain integrin may vary depending on the cell type involved, further enhances the complexity. For instance, $\alpha 2\beta 1$ is a collagen receptor on platelets and a collagen/laminin receptor on endothelial cells [87].

Functional aspects: One function of integrin-mediated adhesion, is the anchorage of cells to basement membranes and to each other. This may be of critical importance in tissue morphogenesis. Integrins also play an important role in cell migration (Fig 3) [72]. For example, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ mediate migration on fibronectin [210] and $\alpha 2\beta 1$ mediates migration on collagen [25]. Importantly, murine embryonic stem cells in which the $\beta 1$ integrin gene was knocked out, show decreased cell migration [43].

Apart from their role in cell adhesion and migration, $\beta 1$-integrins are also involved in the assembly of the ECM. Antibodies against the cytoplasmic domain of the $\beta 1$-subunit have been demonstrated to inhibit fibronectin fibril formation in vivo during development [35]; fibronectin matrix assembly is inhibited in vitro by RGD synthetic peptides [112] and by anti-$\alpha 5$ or anti-$\beta 1$ antibodies [1]; and fibroblasts and melanoma cells can use $\alpha 2\beta 1$ to reorganize and contract hydrated collagen matrices [88].

Finally, yet another, though pathological role for integrins, may be in viral infections. This is illustrated by the reports that $\alpha 2\beta 1$ binds echovirus [9] and that $\alpha v\beta 3$ and $\alpha v\beta 5$ bind adenovirus [208].

Cytoplasmic interactions: The fact that cells can use various integrins for binding to one ligand (Table 1) has important functional consequences. For example, while both $\alpha v\beta 3$ and $\alpha v\beta 5$ bind to vitronectin, only $\alpha v\beta 3$ promotes subsequent migration [97]. Using chimeric integrins, made up of the $\alpha 2$ extracellular and membrane spanning domain and various integrin cytoplasmic domains, it was shown that the cytoplasmic domain plays an important role in such post ligand binding events [24].

The integrin cytoplasmic domains are linked to the cytoskeleton. Following adhesion and spreading of cells, integrins concentrate in focal adhesions (adhesion plaques/adherens junctions) where actin filament bundles are linked to the ECM via integrins (Fig 3). This occurs through indirect linkage, involving talin, vinculin, α-actinin and others [20,50,69,103,131,200]. By using chimeric molecules made up of the cytoplasmic domain of the $\beta 1$ subunit and the IL2r extracellular domain, it has been shown that the $\beta 1$ integrin cytoplasmic part is sufficient for localization in adhesion plaques [94].

Focal adhesions are not the only possible structures for integrin interactions with the cytoskeleton. Integrin $\alpha 6\beta 4$ is part of a distinct epithelial adhesive structure, the hemidesmosome complex [140]. In this complex the extremely long cytoplasmic part of

the β4-chain reaches to the intermediate filaments.

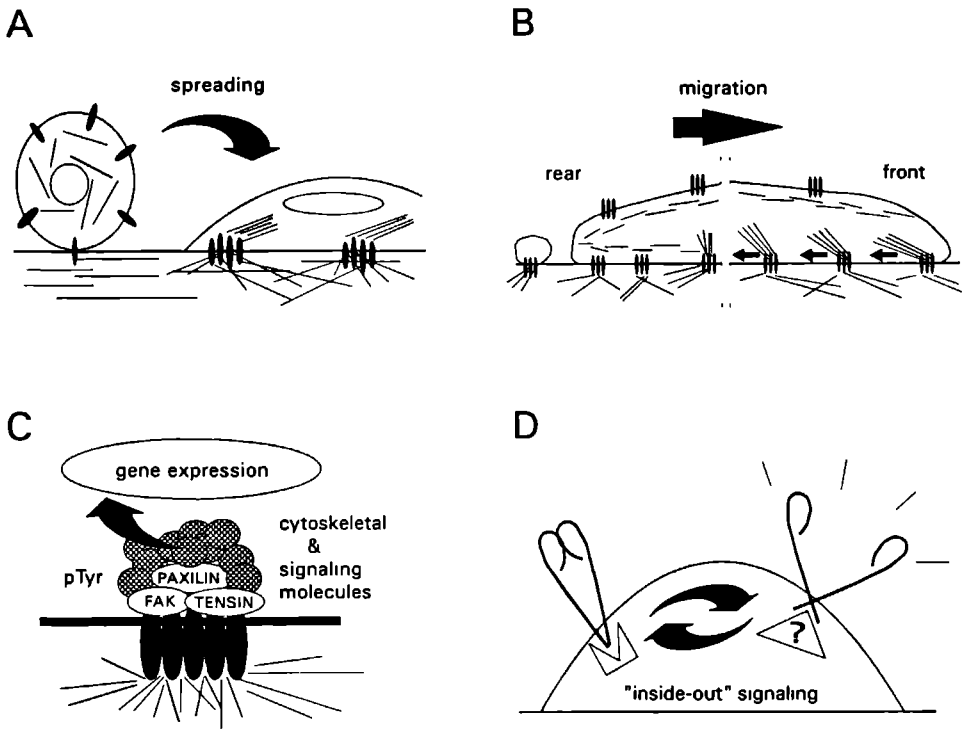


Figure 3. *Integrins in adhesion, migration and signaling events.* ***A:*** *During cell spreading, specialized structures called focal adhesions are formed where clustered integrins link actin filament bundles to the ECM.* ***B:*** *By means of the inward centripetal cytoskeletal stresses (small arrows), these structures provide the traction required for cell migration given that a front-to-rear asymmetry in adhesive contacts exists (e.g. selective downregulation of integrin-cytoskeletal binding strength at the cell rear).* ***C:*** *Clustered integrins in focal adhesions function as a signal transduction unit where cytoskeletal and signaling molecules (e.g. FAK, tensin, and paxilin) are concentrated and phosphorylated (pTyr), ultimately influencing gene expression.* ***D:*** *Unidentified cytoplasmic factors induce conformational changes that control the affinity state of the integrin (inside-out signaling). For references see text.*

Signaling: Concentration of integrins and linkage with the actin filament bundles in focal adhesions are important features in cell migration [72]. In addition, several molecules that

are involved in signal transduction, also assemble in these structures [*see* 28,73,81,155, 159,167]. Recently, integrin-mediated clustering of more than 30 cytoskeletal and signaling molecules was studied [116,117]. It was found that integrin aggregation alone, induces clustering of two molecules, tensin and FAK (focal adhesion kinase). Co-clustering of other cytoskeletal and signaling molecules, as well as activation of the MAP kinase pathway, required additional integrin ligand occupation, tyrosine phosphorylation, and/or actin cytoskeleton integrity.

These initial steps of integrin signaling, are probably at the basis of ECM regulated gene expression (Fig 3) [*see* 28,73,81]. In this way, proliferation and differentiation of cells is not only controlled by soluble growth factors, but also by integrin-mediated adhesion events [122,171,182]. Cyclin A is a key element in adhesion-dependent control of cell proliferation [56], but there is no evidence yet for a link between integrin signaling and cyclin A gene expression. Signal transduction through integrins, neuropeptides, growth factors, and oncogenes may converge in FAK [149,168]. This cytosolic tyrosine kinase can be activated by integrin signaling [57], G-coupled receptor signaling [211], growthfactor receptor signaling [141], and oncogenic $pp60^{src}$ [158].

Finally, integrin-mediated adhesion may also induce signaling events in an alternative fashion. CD47 is a 50 kDa transmembrane molecule with characteristics of a calcium channel that can be expressed in an integrin-bound fashion, hence the name integrin associated protein (IAP). IAP has been shown to be required for RGD stimulation of phagocytosis by PMNs and for integrin-regulated calcium influx in endothelial cells, and it acts in concert with the leukocyte-response-integrin as a signal transduction unit to activate the respiratory burst in phagocytes [19,104,105,160,212].

Regulated activity: In addition to the regulation of adhesion at the level of expression or clustering of integrins, the affinity of individual integrins can be modulated as well. Divalent cations [106], protein kinase C activation [203], lipid factors [66], and even the ligand itself [21], can modulate the activity of integrins. The highly conserved membrane-proximal GFFKR sequence in the α-subunit cytoplasmic tail, has been shown to maintain the default low affinity state of integrins [132]. It is suggested that cytoplasmic signals are required to change the structure of this region, finally resulting in an active conformation of the extracellular part of the integrin. Such a process has been termed inside-out signaling (Fig 3) [73].

Integrins may thus cycle through multiple conformations on the cell surface and antibodies have been generated recognizing or stabilizing active conformations [*see* 37]. A number of antibodies have been generated that enhance ligand binding activity of β1- [92,127,201], β2- [143], and β3-integrins [47]. For the β1-subunit, a small region has been identified that is recognized by both activating and inhibiting antibodies [187]. The authors postulate that binding of activating antibodies to this flexible region leads to an active conformation whereas binding of inhibiting antibodies induces an inactive

conformation.

CD44

Several cell surface molecules that were independently identified over the past decade, later proved to be identical to a human immune cell marker, CD44 [5,62,115]. CD44 is homologous to the cartilage link proteins [54,176]. The 37 kDa transmembrane protein predicted by the cDNA, is post-translationally modified by N- and O-linked glycosilations to an 80-90 kDa glycoprotein [54,176]. In addition, several larger isoforms of CD44 have been described that are generated by alternative splicing of 10 exons (Fig 4) [18,38,58,162,177]. The current view is that CD44 is a family of cell surface glycoproteins, all encoded by a single gene [102]. The 80-90 kDa CD44 species (CD44H) is abundantly expressed on most cell types whereas the larger CD44 isoforms are expressed principally on epithelia and activated leukocytes [4,91,109,169].

This heterogeneity in the CD44 family is probably responsible for the multifunctional character of CD44 on different cell types. CD44H is the receptor for the ECM component hyaluronate (HA) [5,115], whereas data on HA binding by the larger isoform CD44E (CD44v8-10) are conflicting [63,177]. CD44H also binds purified MAdCAM whereas CD44E does not [135]. Conversely, a 180-200 kDa isoform was found to bind fibronectin, whereas CD44H did not [77]. Glycosaminoglycans have been implicated in growthfactor presentation at sites of inflammation [192] and CD44 species containing the v3 exon product are proteoglycans [8,76], which indeed provides these isoforms with the capacity to bind growth factors [8]. Thus, alternative splicing regulates CD44 binding to the ECM-components HA and fibronectin, to the cellular adhesion molecule MAdCAM, and to growth factors. In addition, CD44 has been reported to bind collagen [22].

Intracellularly, CD44H binds ankyrin and this interaction is essential for HA binding [108]. Members of the ERM family (ezrin, radixin, and moesin) have been reported to function as molecular linkers between CD44 and the actin cytoskeleton [199]. These molecules linked a 140 kDa isoform tightly to the cytoskeleton, whereas binding of the 80-90 kDa CD44H molecules was much weaker, despite the fact that both isoforms share the same cytoplasmic domain. It was suggested that this may be related to the tendency of the 140 kDa isoform to cluster.

As adhesion receptors for several ECM components and ligands on endothelial cells, CD44 molecules are involved in cell migration [196], cell aggregation [180], lymphocyte homing [78], and hemopoiesis [114]. Furthermore, CD44 molecules have been implicated in T cell activation [70,172]. In order for a cell adhesion mechanism, involving such broadly distributed molecules as HA and CD44, to be selective, CD44 molecules would not be expected to be constitutively active. Indeed, the HA binding activity of CD44 can be enhanced on T cells during an in vivo immune response [101], and antibodies have been described that activate murine CD44, though the exact mechanism is still unknown [100].

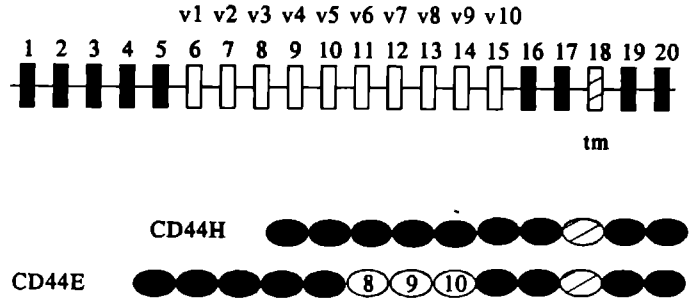


Figure 4. Alternative splicing of CD44 The CD44 gene includes 10 exons that are alternatively spliced (v1-10), resulting either in CD44H that lacks variant exon products, or in isoforms that contain one or several of them, e g CD44E which has v8-10 tm=transmembrane domain

CELL ADHESION RECEPTORS IN MELANOMA AND OTHER CANCERS

In the concept of evolution through random mutations and natural selection during tumor progression, the idea that the adhesive character is among the important acquired properties of the few cells which eventually metastasize, is not new [45,138] The discovery and characterization of the cell adhesion receptors over the past decade, has initiated a search for the aberrant expression or function of these molecules in cancer cells. Immunohistochemical studies have demonstrated changes in the expression of cadherins, integrins, or CD44 isoforms to be related to tumor progression in various human cancers [*see* 2,12,13,52,59,67,82,136,137].

For melanoma, most studies in this field have focussed on integrins [*see* 31,32,121, 152]. Consistent findings are decrease of $\alpha 6\beta 1$, increase of $\alpha 3\beta 1$ and $\alpha 4\beta 1$, and acquirement of $\alpha 5\beta 1$ and $\alpha v\beta 3$ with melanocytic tumor progression (Table 2) In addition, the level of expression of certain integrins has been shown to be related to the metastatic potential of melanoma cell lines [*see* 31]. Of the Ig superfamily, ICAM-1 expression has also been reported to correlate with melanocytic tumor progression in situ [79] In contrast, for CD44, data from a few studies show that it is strongly expressed in all stages of melanocytic tumor progression in situ and in most melanoma cell lines, irrespective of their metastatic potential [40,110,120].

Table 2. Integrin expression in melanocytic tumor progression.

α1β1	α2β1	α3β1	α4β1	α5β1	α6β1	α7β1	αvβ3	α6β4	αvβ5	reference
	⇑									[17,89]
							⇑			[113]
	↑		⇑	↑			⇑			[3]
					⇓					[125]
						⇑				[93]
		⇑								[124]
	↑	↑	⇑		↓		⇑	↑		[120]
↓	⇓		⇑	⇑						[156]
		↑	⇑	⇑	↓		⇑	↓		[33]
	⇑				⇓					[202]
							⇑		⇓	[34]
			⇑							[157]

⇑ or ⇓ indicates that up- or downregulation of those integrins was a major finding, and ↑ or ↓ indicates minor changes. The αIIbβ3/αvβ3 antibody used in [113], is assumed to stain αvβ3 as neither we nor others find αIIbβ3 in human melanoma.

Importantly, for cadherins, integrins, and CD44, direct in vitro and in vivo experimental evidence exists for their role in tumor growth, invasion, and metastasis. For melanoma, this is limited to integrins and CD44 molecules.

Cadherins: Incubation with antibodies to E-cadherin, can induce invasiveness of otherwise non-invasive epithelial cells [7]. Conversely, E-cadherin cDNA transfection into highly invasive, E-cadherin negative carcinoma cells, greatly reduces their invasive capacity [204]. Finally, transfection experiments have shown that expression of E-cadherin in carcinoma cells can reduce their tumorigenicity [126].

Integrins: Overexpression of integrin α5β1 in malignant Chinese hamster ovary (CHO) cells, has been reported to reduce tumorigenicity in nude mice [51]. A role for αlβ2 in lymphoma metastasis has been shown by generating mutants of the highly metastatic TAM2D2 T-cell hybridoma that lacked this integrin. Following intravenous (i.v.) injection, liver colonization was reduced for the mutants [145]. Furthermore, revertants that were obtained by in vivo transplantation, had high αlβ2 expression and were highly metastatic again [144]. A role for the β1 integrins in lung colonization of i.v. injected

carcinoma cells has been demonstrated by an antibody inhibition study [42]. Transfection of non-metastatic rhabdomyosarcoma cells with $\alpha 2$-cDNA, resulting in $\alpha 2\beta 1$-expression and increased adhesion to collagen and laminin, has been observed to induce lung colonization both after i.v. and subcutaneous (s.c.) injection [25].

For human melanoma, $\alpha 5\beta 1$ binding to fibronectin has been shown to induce a mitogenic signal in vitro [122], and αv-cDNA transfection resulting in $\alpha v\beta 3$-expression, has been shown to enhance proliferation in vivo [44]. Furthermore, triggering of $\alpha 5\beta 1$ or $\alpha v\beta 3$ has been reported to induce enhanced metalloprotease expression and invasiveness of human melanoma cells [163,164]. Experimental metastasis of murine melanoma cells after i.v. injection can be inhibited with RGD-containing synthetic peptides, non-peptide mimetics, or disintegrins (RGD-containing peptides isolated from snake venom) [61,71,154,174]. It is not known which RGD-binding integrin is blocked in these experiments but in contrast to human melanoma cells, the B16 murine melanoma cells that were used can express $\alpha IIb\beta 3$ that may be affected [26,68]. In one study, a $\beta 1$-integrin-binding disintegrin was found to inhibit lung colonization of human melanoma cells [197]. The inhibitory effect observed in all these studies may be due to interference with integrin-mediated adhesion during melanoma cell extravasation or subsequent invasion. However, an alternative mode of action might involve inhibition of platelet aggregation or stimulation of natural killer cell activity.

Two reports strongly suggest that the $\alpha 4\beta 1$-VCAM-1 interaction is involved in late stages of melanoma metastasis. First, B16 cells show enhanced lung colonization upon i.v. injection in TNFα-treated mice, and this effect is inhibited by previous injection of antibodies to VCAM-1 [130], and second, the interleukin 1-augmented metastasis of i.v. injected human melanoma cells can be blocked by pretreatment of the melanoma cells with antibodies to $\alpha 4$ [49]. In contrast, by $\alpha 4$-cDNA viral infection, it has been demonstrated that expression of $\alpha 4\beta 1$ inhibits metastasis of s.c. but not i.v. injected B16 cells [139]. Thus, it seems that $\alpha 4\beta 1$ plays a dual role in melanoma progression, inhibiting early stages but facilitating later events of the metastatic cascade.

Antibodies that block attachment to laminin in vitro [205] and peptides from the YIGSR sequence in the laminin B1 chain [75] have been shown to inhibit melanoma cell lung colonization. Furthermore, exposure of cultured cells to laminin before intravenous injection [195] and co-injection of a peptide from the sequence of the laminin A-chain [83] promoted experimental metastasis of melanoma cells. It is not known which laminin receptors are involved (integrins or non-integrins) but $\alpha 6\beta 1$ may be a candidate integrin since an antibody to $\alpha 6$ has been shown to inhibit experimental metastasis of B16 melanoma cells [151]. It may be expected that the antibody would inhibit $\alpha 6\beta 1$ binding to laminin present in the endothelial basement membrane. However, since the same antibody did not inhibit in vitro adhesion to laminin, another function of $\alpha 6\beta 1$ must be affected.

CD44: CD44-Ig fusion proteins have been shown to inhibit tumorigenicity of human

lymphoma cells [185] and cDNA transfection of CD44H but not the larger isoform CD44E, can enhance lymphoma tumorigenicity and lung colonization [184]. Thus, the standard 80-90 kDa CD44 isoform seems to be involved in lymphoma growth and metastasis. A causal role in metastasis of rat prostate carcinoma cells has been demonstrated for CD44 isoforms containing the variant exon v6 product by transfection [58] and antibody inhibition studies [165]. Two v6-containing isoforms, pMeta1 (containing v4,5,6,7) and pMeta2 (containing v6,7) were each found to be sufficient to establish metastatic properties in a non-metastatic cell line [150]. As the HA binding capacity of these isoforms is not different from CD44H which was expressed on the cell line, and as both isoforms lack v3 that is implicated in growth factor binding (*see above*), the mechanism by which they confer metastatic behavior remains to be determined.

For human melanoma, CD44 antibodies that block HA binding have been reported to inhibit tumorigenicity of a human melanoma cell line and inhibit metastasis from a s.c. tumor of that cell line [60]. Furthermore, using a CD44 negative human melanoma cell line (which is very rare), it has been demonstrated by cDNA transfection of wild type and mutant CD44, and by administration of CD44-Ig fusion proteins, that HA binding by CD44 is involved in melanoma tumorigenicity [6].

Thus, for melanoma, the relation of integrin expression with melanocytic tumor progression in situ and with the metastatic potential of cell lines, is extended by findings that integrins can be directly involved in several steps of the metastatic cascade. Even though CD44 molecules are strongly expressed in all stages of melanocytic tumor progression and on most cell lines independent of their metastatic capacity, there is evidence that CD44 may be involved in melanoma growth and metastasis.

SCOPE OF THIS THESIS

For a study on cell adhesion receptors in melanoma, knowledge of the adhesive phenotype of normal melanocytes is important. Therefore, *chapter 2* deals with expression and function of the cell adhesion receptors in cultured melanocytes, maintained either in a highly proliferative state or in a differentiated non-proliferative state. Besides various integrins, melanocytes are shown to express E-cadherin, a cell-cell adhesion molecule that is lost with tumor progression in many epithelial cancers. In order to investigate if E-cadherin is also lost with melanocytic tumor progression, *chapter 3* deals with the expression of this molecule in lesions of the various stages of melanocytic tumor progression in situ, and in melanoma cell lines with different metastatic capacities.

In *chapter 4*, a panel of antibodies is used to determine the pattern of integrin expression in cutaneous melanocytic lesions. The fact that expression of several integrins is found to change dramatically with tumor progression, suggests that integrins may be

involved in melanoma metastasis. As the clinical behavior of uveal melanoma differs from that of cutaneous melanoma, in *chapter 5* the hypothesis that uveal melanoma may express a different pattern of integrins is investigated.

Metastatic melanoma cells penetrate the epidermal and endothelial basement membranes, and integrins that recognize basement membrane components may be important. Therefore, in *chapter 6*, expression and function of $\alpha 6\beta 1$ and $\alpha 2\beta 1$ integrins that bind the basement membrane components laminin and collagen, is investigated in relation to the metastatic potential of melanoma cell lines.

RGD-peptides can inhibit melanoma metastasis, though the integrin(s) involved have not been identified. In *chapter 7*, it is shown that even though $\alpha v\beta 3$ emerges in late stages of tumor progression in situ, two highly metastatic cell lines lack expression of this RGD-binding integrin, suggesting that $\alpha v\beta 3$ is not critical for melanoma metastasis. In contrast, $\alpha 5\beta 1$, another RGD-recognizing integrin that is acquired in late stages of tumor progression (*chapter 4*), is strongly expressed on the highly metastatic cell lines. In *chapter 8*, the mechanism of binding of $\alpha 5\beta 1$ to the RGD-region in fibronectin is investigated. The finding that $\alpha 5\beta 1$ in its high affinity state binds RGD as strong as $\alpha v\beta 3$, suggests that $\alpha 5\beta 1$ may be a possible target for the inhibition of metastasis with RGD peptides. Therefore, in *chapter 9*, various reagents interfering with $\alpha 5\beta 1$ are used in experimental metastasis assays, to investigate the hypothesis that $\alpha 5\beta 1$ may be critical for metastasis of an $\alpha v\beta 3$-negative cell line. The results suggest that this is not the case but a disintegrin that does not bind $\alpha 5\beta 1$, blocks lung colonization and its mode of action is investigated in in vitro assays. In addition, the effect of expression of $\alpha v\beta 3$ on metastasis of this $\alpha v\beta 3$-negative cell line is investigated.

Although CD44 is expressed in all stages of melanocytic tumor progression and in most melanoma cell lines, it has been demonstrated to play a role in growth and metastasis of melanoma cells. It may be that its HA-binding capacity or the availability of HA is critical for metastasis. Therefore, in *chapter 10*, production of HA and other glycosaminoglycans and CD44-mediated adhesion to HA is investigated for human melanoma cell lines in relation to their metastatic potential. As the pattern of CD44 alternative splicing has been related to tumor progression in several malignancies, in *chapters 11 and 12*, expression of CD44 isoforms is investigated in cutaneous and uveal melanoma, both in patients lesions and in cell lines.

REFERENCES

1. Akiyama S, Yamada SS, Chen W, Yamada KM. Analysis of fibronectin receptor function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization J Cell Biol 109, 863-875, 1989.

2. Albelda SM. Role of integrins and other cell adhesion molecules in tumor progression and metastasis.

Lab Invest 68, 4-17, 1993.

3. Albelda SM, Mette SA, Elder DA, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

4. Arch R, Wirth K, Hofmann M, Ponta H, Matzku M, Herrlich P, Zöller M. Participation in normal immune responses of a metastasis-inducing splice variant of CD44. Science 257, 682-685, 1992.

5. Arrufo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 61, 1303-1313, 1990.

6. Bartolazzi A, Peach R, Aruffo A, Stamenkovic I. Interaction between CD44 and hyaluronate is directly involved in the regulation of tumor development. J Exp Med 180, 53-66, 1994.

7. Behrens J, Mareel MM, Van Roy FM, Birchmeier W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 108, 2435-2447, 1989.

8. Bennet KL, Jackson DG, Simon JC, Tanczos E, Pach R, Modrell B, Stamenkovic I, Plowman G, Aruffo A. CD44 isoforms containing exon v3 are responsible for the presentation of heparin-binding growthfactor J Cell Biol 128, 687-698, 1995

9. Bergelson JM, Shepley MP, Chan BMC, Hemler ME, Finberg RW. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science 255, 1718-1720, 1992.

10. Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A, Butcher EC. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 74, 185-194, 1993.

11. Bevilaqua M, Butcher E, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G. Selectins: a family of adhesion receptors. Cell 67, 233, 1991.

12. Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1198, 11-26, 1994.

13. Birchmeier W, Hulsken J, Behrens J. Adherens junction proteins in tumor progression. Cancer Surv 24, 129-140, 1995.

14. Bishop JM. Molecular themes in oncogenesis. Cell 64, 235-248, 1991.

15. Boller K, Vestweber D, Kemler R. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J Cell Biol 100, 327-332, 1985.

16. Briskin M, McEvoy JLM, Butcher EC. MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. Nature 363, 461-463, 1993.

17. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma Int J Cancer 36, 29-35, 1985.

18. Brown TA, Bouchard T, St John T, Wayner E, Carter WG. Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulphate intrinsic membrane proteoglycan with additional exons J Cell Biol 113, 207-221, 1991.

19. Brown E, Hooper L, Ho T, Gresham H. Integrin-associated protein. a 50-kD plasma membrane antigen physically and functionally associated with integrins. J Cell Biol 111, 2785-2794, 1990.

20. Burridge K, Fath K, Kelly T, Nuckolls G, Turner C. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Ann Rev Cell Biol 4,487-525, 1988.

21. Cabanas C, Hogg N. Ligand intercellular adhesion molecule 1 has a necessary role in activation of integrin lymphocyte-associated molecule 1. Proc Natl Acad Sci USA 90, 5838-5842, 1993.

22. Carter WG, Wayner EA Characterization of the class III collagen receptor, a phophorylated transmembrane glycoprotein expressed in nucleated human cells. J Biol Chem 263, 4193-4201, 1988.

23. Cepec KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin Nature 372, 190-193, 1994.

24. Chan BMC, Kassner PD, Chiro JA, Byers R, Kupper TS, Hemler ME. Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell 68, 1051-1060, 1992.

25. Chan BMC, Matsuura N, Takada Y, Zetter BR, Hemler ME. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. Science 251, 1600-1602, 1991.

26. Chang YS, Chen YQ, Timar J, Nelson KK, Grossi IM, Fitzgerald LA, Diglio CA, Honn KV. Increased expression of αIIbβ3 integrin in subpopulations of murine melanoma cells with high lung-colonizing ability. Int J Cancer 51, 445-451,1992.

27. Cheresh DA, Spiro RC. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J Biol Chem 262, 17703-17711, 1987.

28. Clark AE, Brugge JS. Integrins and signal transduction pathways: the road taken. Science 268, 233-239, 1995.

29. Clark WHJr. From the melanocyte to melanoma to tumor biology. Adv Cancer Res 65, 113-140, 1994

30. Cunningham BA, Hemperly BJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science 236, 799-806, 1987.

31. Danen EHJ, Van Muijen GNP, Ruiter DJ. Role of integrins as signal transducing cell adhesion molecules in human cutaneous melanoma. Cancer Surv 24, 43-65, 1995.

32. Danen EHJ, Van Muijen GNP, Ten Berge PJM, Ruiter DJ. Integrins and melanoma progression Recent Results Cancer Res 128, 119-132, 1993.

33. Danen EHJ, Ten Berge PJM, Van Muijen GNP, Van 't Hof-Grootenboer AE, Brōcker AB, Ruiter DJ Emergence of α5β1 fibronectin- and αvβ3 vitronectin-receptor expression in melanocytic tumor progression Histopathol 24, 249-256, 1994.

34. Danen EHJ, Jansen KFJ, Van Kraats AA, Cornelissen IMHA, Ruiter DJ, Van Muijen GNP αv-Integrins in human melanoma: gain of αvβ3 and loss of αvβ5 are related to tumor progression in situ but not to metastatic capacity of cell lines in nude mice. Int J Cancer 61, 491-496, 1995.

35. Darribère T, Guida K, Larjava H, Johnson KE, Yamada KM, Thiery JP, Boucaut JC. In vivo analysis of integrin β1 subunit function in fibronectin matrix assembly. J Cell Biol 110, 1813-1823, 1990.

36. De Fougerolles AR, Springer TA. Intercellular adhesion molecule 3, a third counterreceptor for leukocyte function associated antigen 1 on resting lymphocytes. J Exp Med 175, 185-190, 1992.

37. Diamond MS, Springer TA. The dynamic regulation of integrin adhesiveness. Curr Biol 4, 506-517, 1994.

38. Dougherty GJ, Lansdorp PM, Cooper DL, Humphries RK. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hematopoietic cells. J Exp Med 174, 1-5, 1991.

39. D'Souza SE, Haas TA, Piotrowicz RS, Byers-Ward V, McGrath DE, Soule HR, Cierniewski C, Plow EF, Smith JW. Ligand and cation binding are dual functions of a discrete segment of the integrin beta 3 subunit. cation displacement is involved in ligand binding. Cell 79, 659-667, 1994.

40. East JA, Mitchell SD, Hart IR. Expression and function of the CD44 glycoprotein in melanoma cell lines. Melanoma Res 3, 341-346, 1993.

41. Elices MJ, Osborn L, Tanaka Y, Crouse C, Luhowsky S, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a distinct site from the VLA4/fibronectin binding site Cell 60, 577-584, 1990.

42. Elliot BE, Ekblom P, Pross H, Nieman A, Rubin K. Anti beta 1 integrin IgG inhibits pulmonary macrometastasis and the size of micrometastases from a murine mamary carcinoma. Cell Adh Comm 1, 319-332, 1994.

43. Fassler R, Pfaff M, Murphy J, Noegel AA, Johansson S, Timpl R, Albrecht R. Lack of $\beta 1$ integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocyts. J Cell Biol 128, 979-988, 1995.

44. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.

45. Fidler I, Hart I. Biological diversity in metastatic neoplasms: origins and implications. Science 217, 998-1003, 1982.

46. Fidler IJ, Radinsky R. Genetic control of cancer metastasis. J Natl Cancer Inst 82, 166-168, 1990.

47. Frehlinger AL, Du XP, Plow EF, Ginsberg MH. Monoclonal antibodies to ligand-occupied conformers of integrin αIIbβ3 (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function J Biol Chem 266, 17106-17111, 1991.

48. Garrod DR. Desmosomes and hemidesmosomes. Curr Opin Cell Biol 5, 30-40, 1993.

49. Garofalo A, Chirivi RGS, Foglieni C, Pigott R, Mortarini R, Martin-Padura I, Anichini A, Gearing AJ, Sanchez-Madrid F, Dejana E, Giavazzi R. Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastasis. Cancer Res 55, 414-419, 1995.

50. Geiger B, Ginsberg D. The cytoplasmic domain of adherens type junctions. Cell Motil Cytoskeleton 20, 1-6, 1991.

51. Giancotti FG, Ruoslahti E. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells Cell 60, 849-459, 1990.

52. Giancotti FG, Mainiero F. Integrin-mediated adhesion and signalling in tumorigenesis. Biochim Biophys Acta 1198, 47-64, 1994

53. Ginsberg MH, Loftus JC, and Plow EF. Cytoadhesins, integrins, and platelets. Thromb Haemost 59, 1-6, 1988.

54. Goldstein LA, Zhou DFH, Picker LJ, Minty CN, Bargtze RF, Ding JF, Butcher EC. A human lymphocyte homing receptor, the Hermes antigen is related to cartilage proteoglycan core and link protein Cell 56, 1063-1072, 1989.

55. Grunwald GB The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules Curr Opin Cell Biol 5, 797-805, 1993.

56. Guadagno TM, Ohtsubo M, Roberts JM, Assoian RK. A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262, 1572-1575, 1993.

57. Guan J-L, Shalloway D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 358, 690-692, 1992.

58. Günthert U, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65, 13-24, 1991.

59. Günthert U, Stauder R, Mayer B, Terpe HJ, Finke L, Friedrichs K Are CD44 isoforms involved in human tumour progression? Cancer Surv 24, 19-42, 1995.

60. Guo Y, Ma J, Wang J, Che X, Narula J, Bigby M, Wu M, Sy MS. Inhibition of melanoma growth and metastasis in vivo by anti-CD44 monoclonal antibody. Cancer Res 54, 1561-1565, 1994.

61. Hardan I, Weiss L, Hershkoviz R, Freenspoon N, Alon R, Cahalon L, Reich S, Slavin S, Lider O. Inhibition of metastatic cell colonization in murine lungs and tumor-induced morbidity by non-peptidic arg-gly-asp mimetics. Int J Cancer 55, 1023-1028, 1993.

62. Haynes BF, Telen MJ, Hale LP, Denning SM. CD44-a molecule involved in leucocyte adherence and

T-cell activation. Immunol Today 10, 423-428, 1989.
63. He Q, Lesley J, Hyman R, Ishihara K, Kincade PW. Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. J Cell Biol 119, 1711-1719, 1992.
64. Herlyn ME, Clark WH, Rodeck U, Mancianti ML, Jambrosic J, Koprowski H. Biology of tumor progression in human melanocytes. Lab Invest 56, 461-474, 1987.
65. Herrlich P, Zoller M, Pals ST, Ponta H. CD44 splice variants· metastases meet lymphocytes Immunol Today 14, 395-399, 1993.
66. Hermanovski-Vosatka A, Van Strijp JAG, Swiggard WJ, Wright SD. Integrin modulating factor-1·a lipid that alters the function of leucokyte integrins. Cell 68, 341-352, 1992.
67. Herrlich P, Zöller M, Pals ST, Ponta H. CD44 splice variants: metastases meet lymphocytes. Immunol Today 14, 395-399, 1993.
68. Honn KV, Chen YQ, Timar J, Onoda JM, Hatfield JS, Fligiel SEG, Steinert BW, Diglio CA, Grossi IM, Nelson KK, Taylor JD. αIIbβ3 integrin expression and function in subpopulations of murine tumors. Exp Cell Res 201, 23-32, 1992.
69. Horwitz A, Duggan K, Buck C, Beckerle M, Burridge K. Interaction of plasma membrane fibronectin receptor with talin - a transmembrane linkage. Nature 320, 531-533, 1986.
70. Huet S, Groux H, Caillou B, Valentin H, Prieur M, Bernard A. CD44 contributes to T cell activation. J Immunol 143, 798-801, 1989.
71. Humphries MJ, Olden K, Yamada KM. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 233, 467-470, 1986.
72. Huttenlocher A, Sandborg RR, Horwitz AF. Adhesion in cell migration. Curr Opinion Cell Biol, in press, 1995.
73. Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion Cell 69, 11-25, 1992
74. Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations, and targetting of cells and axons. Cell 68, 303-322, 1992.
75. Iwamoto Y, Robey FA, Graf J, Sasaki M, Kleinman HK, Yamada Y, Martin GR. YIGSR, a synthetic laminin pentapeptide, inhibits metastasis formation. Science 238, 1132-1134, 1987.
76. Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, Whittle N. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon J Cell Biol, 128, 673-685, 1995.
77. Jalkanen S, Jalkanen M Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J Cell Biol 116, 817-825, 1992.
78. Jalkanen S, Steere AC, Fox RI, Butcher EC. A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. Science 233, 556-558, 1986.
79. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmuller G. De novo expression of intercellular adhesion molecule 1 in primary and metastatic melanoma lesions. Proc Natl Acad Sci USA 86, 641-644, 1989.
80. Juan M, Vinas O, Pito-Ottin MR, Places L, Martinez-Caseres E, Barcello J, Miralles A, Vitella R, de la Fuente MA, Vives J, Jague J, Gaya A. CD50 (intercellular adhesion molecule 3) stimulation induces calcium mobilisation and tyrosine phophorylation through p59fyn and p56lck in Jurkat T cell line J Exp Med 179, 1747-1756, 1994.
81. Juliano RL, Haskill S. Signal transduction from the extracellular matrix J Cell Biol 120, 577-585, 1993.
82. Juliano RL, Varner JA. Adhesion molecules in cancer: the role of integrins Curr Opinion Cell Biol 5, 812-818, 1993.

83. Kanemoto T, Reich R, Royce L, Greatorex D, Adler SH, Shiraishi N, Martin GR, Yamada Y, Kleinman HK. Identification of an amino acid sequence from the laminin A chain that stimulates metastasis and collagenase IV production. Proc Natl Acad Sci USA 87, 2279-2283, 1990.

84. Karpatkin S, Pearlstein E, Ambrogio C, Coller BS. Role of adhesive proteins in platelet tumor interactions in vitro and metastasis formation in vivo. J Clin Invest 81, 1012-1019, 1988.

85. Kaufmann R, Frosch D, Westphal C, Weber L, Klein CE. Integrin VLA-3: Ultrastructural localization at cell-cell contact sites of human cell cultures. J Cell Biol 109, 1807-1815, 1989.

86. Kintner C. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell 69, 225-236, 1992.

87. Kirchhofer D, Languino LR, Ruoslahti E, Pierschbacher MD. $\alpha 2\beta 1$ Integrins from different cell types show different binding specificities. J Biol Chem 265, 615-618, 1990.

88. Klein CE, Dressel D, Steinmayer T, Mauch C, Eckes B, Krieg T, Bankert RB, Weber L. Integrin $\alpha 2\beta 1$ is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. J Cell Biol 115, 1427-1436, 1991.

89. Klein CE, Steinmeyer T, Kaufmann D, Weber L, Brocker E-B. Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96, 281-284, 1991.

90. Koch PJ, Franke WW. Desmosomal cadherins: another growing multigene family of adhesion molecules. Curr Opin Cell Biol 6, 682-687, 1994.

91. Koopman G, Heider KL, Horst E, Adolf GR, Van den Berg F, Ponta H, Herrlich P, Pals ST. Activated human lymphocytes and aggressive non-Hodgkin lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 177, 897-904, 1993.

92. Kovach NL, Carlos TM, Yee E, Harlan JM. A monoclonal antibody to $\beta 1$ integrin (CD29) stimulates VLA-dependent adherence of leucocytes to human umbilical vein endothelial cells and matrix components J Cell Biol 116, 499-509, 1992.

93. Kramer RH, Vu MP, Cheng YF, Ramos DM, Timpl R, Waleh N. Laminin-binding integrin $\alpha 7\beta 1$: functional characterization and expression in normal and malignant melanocytes. Cell Regul 2, 805-817, 1991.

94. La Flamme SE, Akiyama SK, Yamada KM. Regulation of fibronectin receptor distribution. J Cell Biol 117, 437-447, 1992.

95. Larjava H, Peltonen J, Akiyama SK, Yamada SS, Gralnick HR, Uitto J, Yamada KM Novel function for $\beta 1$ integrins in keratinocyte cell-cell interactions. J Cell Biol 110, 803-815, 1991.

96. Lasky LA, Rosen SD The selectins carbohydrate binding adhesion molecules of the immune system. In: J Gallin, I Goldstein, R Snyderman (eds) Inflamation: basic principles and clinical correlates, pp 103-117. Raven Press, New York, 1992.

97. Leavesly DI, Ferguson GD, Wayner EA, Cheresh DA. Requirement of the integrin $\beta 3$ subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J Cell Biol 117, 1101-1107, 1992.

98. Legan PK, Collins JE, Garrod DR. The molecular biology of desmosomes and hemidesmosomes: what's in a name? BioEssays 14, 385-393, 1992.

99. Lenter M, Vestweber D. The integrin chains $\beta 1$ and $\alpha 6$ associate with the chaperone calnexin prior to integrin assembly. J Biol Chem 269, 12263-12208, 1994.

100. Lesley J, He Q, Miyake K, Hamann A, Hyman R, Kinkade PW. Requirements for hyaluronic acid binding by CD44. a role for the cytoplasmic domain and activation by antibody. J Exp Med 175, 257-266, 1992

101. Lesley J, Howes N, Perschl A, Hyman R. Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. J Exp Med 180, 383-387, 1994.

102. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. Adv Immunol

54, 271-335, 1993.

103. Lewis JM, Schwartz MA. Mapping in vivo associations of cytoplasmic proteins with integrin $\beta 1$ cytoplasmic domain mutants. J Cell Sci 6, 151-160, 1995.

104. Lindberg FP, Gresham HD, Schwarz E, Brown E. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in $\alpha v\beta 3$-dependent ligand biding. J Cell Biol 123, 485-596, 1993.

105. Lindberg FP, Lublin DM, Telen MJ, Veile RA, Miller YE, Donnis-Keller H, Brown EJ Rh-related antigen CD47 is the signal-transducer integrin-associated protein. J Biol Chem 269, 1567-1570, 1994

106. Loftus JC, O'Toole TE, Plow EF, Glas A, Frelinger AL, Ginsberg MH. A $\beta 3$ integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249, 915-918, 1990.

107. Loftus JC, Smith JW, Ginsberg MH. Integrin-mediated cell adhesion: the extracellular face. J Biol Chem 269, 25235-25238, 1994.

108. Lokeshwar VB, Fregien N, Bourguignon LYW. Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J Cell Biol 126, 1099-1109, 1994.

109. MacKay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Günthert U Expression and modulation of CD44 variant isoforms in humans. J Cell Biol 124, 71-82, 1994.

110. Manten-Horst E, Danen EHJ, Smit L, Snoek M, LePoole C, Van Muijen GNP, Pals ST, Ruiter DJ Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential. Int J Cancer 64, 182-188, 1995

111. Marcantonio EE, Hynes RO. Antibodies to the conserved cytoplasmic domain of the integrin $\beta 1$ subunit react with proteins in vertebrates, invertebrates and fungi. J Cell Biol 106, 1765-1772, 1988

112. McDonald JA, Quade BJ, Broekelmann TJ, LaChance R, Forsman K, Hasegawa E, Akiyama S. Fibronectin's cell-ahesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J Biol Chem 262, 2957-2967, 1987.

113. McGregor BC, McGregor JL, Weiss LM, Wood GS, Hu CH, Warnke RA. Presence of cytoadhesins (IIb-IIIa-like glycoproteins) on human metastatic melanomas but not on benign melanocytes Am J Clin Pathol 92, 495-499, 1989.

114. Miyake K, Medina KL, Hayashi SI, Ono S, Hamaoka T, Kincade PW. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow culture. J Exp Med 171, 477-488, 1990

115. Miyake K, Underhill CB, Lesley J, Kincade PW. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J Exp Med 172, 69-75, 1990.

116. Miyamoto S, Akiyama SK, Yamada KM. Synergystic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 266, 1719-1723, 1995.

117. Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol, in press, 1995

118. Mignatti P, Rifkin D Biology and biochemistry of proteinases in tumor invasion Physiol Rev 73, 161-195, 1993.

119. Momburg F, Koch S. Selective loss of the $\beta 2$-microglobulin mRNA in human colon carcinoma J Exp Med 169, 309-314, 1989.

120. Moretti S, Martini L, Berti E, Pinzi C, Gianotti B. Adhesion molecule profile and malignancy of melanocytic lesions. Melanoma Res 3, 235-239, 1993.

121. Mortarini R, Anichini A. From adhesion to signalling: roles of integrins in the biology of human melanoma. Melanoma Res 3, 87-97, 1993.

122. Mortarini R, Gismondi A, Santoni A, Parmiani G, Anichini A. Role of the $\alpha 5\beta 1$ integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. Cancer Res 52, 4499-4506, 1992.

123. Nagafuchi A, Takeichi M. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J 7, 3679-3684, 1988.

124. Natali PG, Nicotra MR, Bartolazzi A, Cavaliere R, Bigotti A. Integrin expression in cutaneous malignant melanoma: association of the $\alpha 3/\beta 1$ heterodimer with tumor progression. Int J Cancer 54, 68-72, 1993.

125. Natali PG, Nicotra MR, Cavaliere R, Giannarelli D, Bigotti A. Tumor progression in human malignant melanoma is associated with changes in $\alpha 6/\beta 1$ laminin receptor. Int J Cancer 49, 168-172, 1991.

126. Navaro P, Gomez M, Pizzaro A, Gamallo C, Quintanilla M, Cano A. A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. J Cell Biol 115, 517-533, 1991.

127. Neugebauer KM, Reichardt LF. Cell surface regulation of beta 1-integrin activity on developing retinal neurons. Nature 350, 68-71, 1991.

128. Nose A, Nagafuchi A, Takeichi M. Expressed recombinant cadherins mediate cell sorting in model systems Cell 54, 993-1001, 1988.

129. Nose A, Tsuji K, Takeichi M. Localization of specificity determining sites in cadherin cell adhesion molecules. Cell 61, 147-155, 1990.

130. Okahara H, Yagita H, Miyake K, Okumura K. Involvement of very late antigen (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor α enhancement of experimental metastasis. Cancer Res 54, 3233-3236, 1994.

131. Otey C, Pavalko F, Burridge K. An interaction between α-actinin and the $\beta 1$ integrin subunit in vitro J Cell Biol 111, 721-729, 1990.

132. O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ, Ginsberg MH. Integrin cytoplasmic domains mediate inside-out signal transduction. J Cell Biol 124, 1047-1059, 1994.

133. Ozawa M, Baribault H, Kemler R. The cytplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J 8, 1711-1717, 1989.

134. Pauli BU, Knudson W. Tumor invasion: a consequence of destructive and compositional matrix alterations. Hum Pathol 19, 628-639, 1989.

135. Picker LJ, Nakache M, Butcher EC. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. J Cell Biol 109, 927-938, 1989

136. Pignatelli M, Stamp G. Integrins in tumor development and spread. Cancer Surv 24, 113-127, 1995

137. Ponta H, Sleeman J, Herrlich P Tumor metastasis formation: cell-surface proteins confer metastasis-promoting or -suppressing properties. Biochim Biophys Acta 1198, 1-10, 1994.

138. Poste G, Fidler IJ The pathogenesis of cancer metastasis. Nature 283, 139-146, 1980.

139. Qian F, Vaux DL, Weissman I Expression of the integrin $\alpha 4\beta 1$ on melanoma cells can inhibit the invasive stage of metastasis formation. Cell 77, 335-347, 1994

140. Quaranta V, Jones JCR. The internal affairs of an integrin. Trends Cell Biol 1, 2-4, 1991

141. Rankin S, Rozengurt E PDGF modulation of focal adhesion kinase p125FAK and paxillin tyrosine phosphorylation in Swiss 3T3 cells, bell-shaped dose response and cross-talk with bombesin J Biol Chem 269, 704-710, 1994.

142. Rice GE, Bevilaqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246, 1303-1306, 1989.

143. Robinson MK, Andrew D, Rosen H, Brown D, Ortlepp S, Stephens P, Butcher EC. Antibody against the leu-cam β-chain (CD 18) promotes both LFA-1 and CR3-dependent adhesion events. J Immunol 148, 1080-1058, 1992.

144. Roossien FF, de Kuiper PE, de Rijk D, Roos E. Invasive and metastatic capacity of revertants of LFA-1-deficient mutant T-cell hybridomas. Cancer Res 50, 3509-3513, 1990.

145. Roossien FF, de Rijk D, Bikker A, Roos E. Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. J Cell Biol 108, 1979-1985, 1989

146. Rosen SD, Bertozzi CR The selectins and their ligands. Curr Opin Cell Biol 6, 663-673, 1994.

147. Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol 137, 1270-1276, 1986.

148. Rothlein R, Kishimoto TK, Mainolfi E. Crosslinking of ICAM-1 induces co-signalling of an oxidative burst from mononuclear leucocytes. J Immunol 152, 2488-2495, 1994.

149. Rozengurt E. Convergent signalling in the action of integrins, neuropeptides, growthfactors and oncogenes. Cancer Surv 24, 81-96, 1995.

150. Rudy W, Hofmann M, Schwarz-Albiez R, Zoller M, Heider K-H, Ponta H, Herrlich P. The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants each one individually suffices to confer metastatic behavior. Cancer Res 53, 1262-1268, 1993

151. Ruiz P, Dunon D, Sonnenberg A, Imhof BA. Suppression of mouse melanoma metastasis by EA-1, a monoclonal antibody specific for alpha(6)-integrins. Cell Adh Commun 1, 67-81, 1993

152. Runger TM, Klein CE, Becker JC, Brocker EB. The role of genetic instability, adhesion, cell motility, and immune escape mechanisms in melanoma progression. Curr Opin Oncol 6, 188-196,1994

153. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion. Science 283, 491-497, 1987.

154. Saiki I, Iida J, Murata J, Ogawa R, Nishi N, Sugimura K, Tokura S, Azuma I. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. Cancer Res 49, 3815-3822, 1989.

155. Sastry SK, Horwitz AF. Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signalling Curr Opin Cell Biol 5, 819-831, 1993.

156. Schadendorf D, Gawlik C, Haney U, Ostmeier H, Suter L, Czarnetzki BM. Tumor progression and metastatic behavior in vivo correlates with integrin expression on melanocytic tumors. J Pathol 170, 429-434, 1993.

157. Schadendorf D, Heidel J, Gawlik C, Suter L, Czarnetzki BM. Association with clinical outcome of expression of VLA-4 in primary melanoma as well as P-selectin and E-selectin on intratumoral vessels. J Natl Cancer Inst 87, 366-371, 1995.

158. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 89, 5192-5196, 1992.

159. Schaller MD, Parsons JT. Focal adhesion kinase and associated proteins. Curr Opin Cell Biol 6, 705-710, 1994.

160. Schwarz MA, Brown E, Fazeli B. A 50-kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. J Biol Chem 27, 19931-19934, 1993.

161. Schwarz MA, Owaribe K, Kartenbeck J, Franke WW. Desmosomes and hemidesmosomes: constitutive molecular components. Annu Rev Cell Biol 6, 461-491, 1990.

162. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell IJ. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons Proc Natl Acad Sci USA 89, 12160-12164, 1992.

163. Seftor REB, Seftor EA, Gehlsen KR, Stetler-stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC Role of the $\alpha v\beta 3$ integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 89, 1557-1561, 1992.

164. Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC. The 72-kDa type IV collagenase is modulated via differential expression of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins during melanoma cell invasion Cancer

Res 53, 3411-3415, 1993.

165. Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzku S, Zöller M. Prevention of tumor metastasis formation by anti-variant CD44. J Exp Med 177, 443-455, 1993.

166. Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand J-F, Als-Nielsen J, Colman DR, Hendrickson WA. Structural basis of cell-cell adhesion by cadherins. Nature 374, 327-337, 1995.

167. Shattil SJ, Ginsberg MH, Brugge JS. Adhesive signaling in platelets. Curr Opin Cell Biol 6, 695-704, 1994.

168. Shattil SJ, Haimovich B, Cunningham M, Lipfert L, Parsons JT, Ginsberg MH, Brugge JS. Tyrosine phosphorylation of pp125FAK in platelets requires coordinated signalling through integrin and agonist receptors. J Biol Chem 269, 14738-14745, 1994.

169. Sherman L, Sleeman J, Herrlich P, Ponta H. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. Curr Opin Cell Biol 6, 726-733, 1994.

170. Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells Immunol Today 13, 106-110, 1992.

171. Shimizu Y, Van Seventer GA, Horgan KJ, Shaw S. Costimulation of proliferative responses of resting CD4+ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin J Immunol 1456, 59-64, 1990.

172. Shimizu Y, Van Seventer GA, Siraganian R, Wahl L, Shaw S. Dual role of the CD44 molecule in T cell adhesion and activation J Immunol 143, 2457-2463, 1989.

173. Simmons DL. The role of ICAM expression in immunity and desease. Cancer Surv 24, 141-155, 1995

174. Soszka T, Knudsen KA, Beviglia L, Rossi C, Poggi A, Niewiarowski S. Inhibition of murine melanoma cell-matrix adhesion and experimental metastasis by albolabrin, an RGD-containing peptide isolated from the venom of Trimeresurus albolabris. Exp Cell Res 196, 6-12, 1991.

175. Springer TA. Traffic signals for lymphocyte recirculation and leucocyte emigration the multistep paradigm. Cell 76, 301-314, 1994.

176. Stamenkovic I, Amiot M, Pesando JM, Seed B. A lymphocyte molecule implicated in lymphocyte homing is a member of the cartilage link protein family. Cell 56, 1057-1062, 1989.

177. Stamenkovic I, Aruffo A, Amiot M, Seed B. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J 10, 343-348, 1991

178. Staunton DE, Dustin ML, Springer TA. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-2. Nature 339, 361-364, 1989.

179. Steegmaler M, Levinovitz A, Isenmann S, Borges E, Lenter M, Kocher HP, Kleuser B, Vestweber D The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growthfactor Nature 373, 615-620, 1995.

180. St John T, Meyer J, Idzerda R, Gallatin M. Expression of CD44 confers a new adhesive phenotype on transfected cells. Cell 60, 45-52, 1990.

181. Sriramarao P, Steffner P, Gehlsen KR Biochemical evidence for a homophylic interaction of the $\alpha 3\beta 1$ integrin J Biol Chem 268, 22036-22041, 1994.

182. Sugahara H, Kanukura Y, Furitsu T, Ishihara K, Oritani K, Ikeda H, Kitayama H, Ishikama J, Hashimoto K, Kanayama Y, Matsuzawa Y. Induction of programmed cell death in human hematopoietic cell lines by fibronectin via its interaction with very late antigen 5. J Exp Med 179, 1757-1766, 1994

183. Suzuki S, Sano K, Tanihara H. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue Cell Regul 2, 261-270, 1991.

184. Sy M-S, Guo Y-J, Stamenkovic I Distinct effects of two CD44 isoforms on tumor growth in vivo. J Exp Med 174, 859-866, 1991.

185. Sy M-S, Guo Y-J, Stamenkovic I. Inhibition of tumor growth in vivo with a soluble CD44-immunoglobulin fusion protein. J Exp Med 176, 623-627, 1992.

186. Symington BE, Takada Y, Carter WG. Interaction of integrins $\alpha 3\beta 1$ and $\alpha 2\beta 1$: potential role in keratinocyte intercellular adhesion J Cell Biol 120, 523-535, 1993.

187. Takada Y Puzon W. Identification of a regulatory region of integrin $\beta 1$ subunit using activating and inhibiting antibodies. J Biol Chem 268, 17597-17601, 1993.

188. Takeichi M. The cadherins. cell-cell adhesion molecules controlling animal morphogenesis. Development 102, 639-655, 1988.

189. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion Annu Rev Biochem 59, 237-252, 1990.

190. Takeichi M. Cadherin cell adhesion receptors as a morphogenic regulator. Science 251, 1451-14555, 1991.

191. Takeichi M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol 5, 806-811, 1993.

192. Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist S, Shaw S. T cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. Nature 361, 79-82, 1993.

193. Tedder TF, Steeber DA, Chen A, Engel P. The selectins: vascular adhesion molecules FASEB J 9, 866-873, 1995.

194. Terranova VP, Liotta LA, Russo RG, Martin GR. Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res 42, 2265-2269, 1982.

195. Terranova VP, Williams JE, Liotta LA, Martin GR. Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. Science 226, 982-985, 1984.

196. Thomas L, Byers HR, Vink J, Stamenkovic I. CD44H regulates tumor migration on hyaluronate-coated substrate. J Cell Biol 118, 971-977, 1992.

197. Trikha M, De Clerck YA, Markland FS. Contortrostatin, a snake venom disintegrin, inhibits $\beta 1$ integrin-mediated human metastatic melanoma cell adhesion and blocks experimental metastasis Cancer Res 54, 4993-4998, 1994.

198. Tsukita S, Itoh M, Nagafuchi A, Yonemura S Submembranous junctional plaque proteins include potential tumor suppressor molecules. J Cell Biol 123, 1049-1053, 1993.

199. Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskelotons. J Cell Biol 126, 391-401, 1994.

200. Turner CE, Burridge K. Transmembrane molecular assemblies in cell-extracellular matrix interactions Curr Oppin Cell Biol 3, 849-853, 1991.

201. Van de Wiel-van Kemenade P, van Kooyk Y, de Boer AJ, Huijbers RJF, Weder P, van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

202. Van Duinen CM, Van den Broek LJCM, Vermeer BJ, Fleuren GJ, Bruijn JA The distribution of cellular adhesion molecules in pigmented skin lesions. Cancer 73, 2131-2139, 1994.

203. Van Kooyk Y, van de Wiel-van Kemenade P, Weder P, Kuijpers TW, Figdor CG. Enhancement of LFA-1 mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes Nature 342, 811-813, 1989.

204. Vleminckx K, Vakaet L, Mareel Jr M, Fiers W, Van Roy F. Genetic manipulation of cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 66, 107-119, 1991

205. Vollmers HP, Imhof BA, Braun S, Waller CA, Schirrmacher V, Birchmeier W Monoclonal

antibodies which prevent experimental lung metastases. FEBS lett 172, 17-20, 1984.

206. Weinstat-Saslow D, Steeg PS. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. FASEB J 8, 401-407, 1994.

207. Weterman MAJ, Van Muijen GNP, Bloemers HPJ, Ruiter DJ. Molecular markers of melanocytic tumor progression. Lab Invest 70, 593-607, 1994.

208. Wickham TJ, Mathial P, Cheresh DA, Nemerow GR. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, receptors for adenovirus internalization. Cell 73, 309-319, 1992.

209. Williams AF, Barklay AN. The immunoglobulin superfamily: domains for cell surface recognition. Annu Rev Immunol 6, 381-405, 1988.

210. Wu CY, Fields AJ, Kapteijn BAE, McDonald JA. The role of $\alpha 4\beta 1$ integrin in cell motility and fibronectin matrix assembly. J Cell Sci 108, 821-829, 1995.

211. Zachary I, Sinnet-Smith J, Rozengurt E. Bombesin, vasopressin and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells: identification of a novel tyrosine kinase as a major substrate. J Biol Chem 266, 24126-24133, 1992.

212. Zhou M, Brown EJ. Leukocyte response integrin and integrin-associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. J Exp Med 178, 1165-1174, 1993.

Chapter 2

Loss of adhesion to basement membrane components but not to keratinocytes in proliferating melanocytes

Eur J Cell Biol, in press

Loss of adhesion to basement membrane components but not to keratinocytes in proliferating melanocytes

Erik HJ Danen[1], Kees FJ Jansen[1], C Eberhard Klein[2], Nico PM Smit[3], Dirk J Ruiter[1], and Goos NP van Muijen[1]

[1]Department of Pathology, University Hospital, Nijmegen, The Netherlands, [2]Department of Dermatology, University Hospital, Würzburg, Germany, and [3]Department of Dermatology, Academic Medical Center, Amsterdam, The Netherlands

We studied the adhesive characteristics of melanocytes, cultured either in the presence of the mitogen PMA that keeps them in a proliferative state, or in the absence of PMA allowing them to differentiate. On proliferating melanocytes, several integrins, ICAM-1, E-cadherin, and CD44 were expressed. In the absence of PMA, proliferation was arrested, melanin synthesis increased, and the morphology of the melanocytes became more spreaded. Under these conditions, expression of integrins $\alpha 3\beta 1$ and $\alpha 5\beta 1$ decreased whereas expression of $\alpha 2\beta 1$, $\alpha 4\beta 1$, and $\alpha 6\beta 1$ increased. No changes were observed for any of the other adhesion molecules. Immunoprecipitations from metabolically labeled cells confirmed the shift in integrin expression at the level of biosynthesis. The increased surface expression of $\alpha 2\beta 1$ and $\alpha 6\beta 1$ in the absence of PMA was accompanied by an induction of adhesion to basement membrane components collagen and laminin through these integrins. Integrin $\alpha 5\beta 1/\alpha v\beta 3$-mediated adhesion to fibronectin, CD44-mediated adhesion to hyaluronate, and E-cadherin/$\beta 1$-integrin-mediated adhesion to keratinocytes were not affected by PMA. These findings indicate that by selective modulation of the expression of adhesion molecules, adhesion to components of the basement membrane is reduced in proliferating melanocytes whereas adhesion to keratinocytes is maintained. Similar events may be involved in melanocyte proliferation and migration during wound healing and initial steps of melanocytic tumor progression.

INTRODUCTION

Melanocytes are neural crest-derived cells that are mainly situated in the basal cell layer of the epidermis where they make contact with the basement membrane and with adjacent keratinocytes. A single melanocyte distributes pigment to 25 to 35 keratinocytes through its dendrites, and such a cluster has been termed an "epidermal melanin unit" [12]. By production and transfer of melanin to keratinocytes, melanocytes protect keratinocytes from damage by UV radiation. In vitro, the morphology of melanocytes is controlled by contact with extracellular matrix (ECM) components [13], and keratinocytes control differentiation and proliferation of melanocytes [11,38]. In vivo, normal melanocytes do not proliferate unless stimulated by environmental factors such as UV radiation or during processes such as wound healing [38]. Adhesive contacts with surrounding keratinocytes and the underlaying basement membrane may help to conserve the non-proliferative state and characteristic morphology of melanocytes.

Molecules that mediate cell adhesion can be grouped in distinct families based on structural homologies between the members [17]. *Integrins* are $\alpha\beta$-heterodimeric cell surface glycoproteins that make heterophilic contacts with ECM proteins or with counterreceptors on other cells. Their cytoplasmic domains contact the cytoskeleton and they act as signaling receptors. Binding of integrins to their ligands can influence gene expression and control proliferation of cells [16]. Members of the *immunoglobulin (Ig) superfamily* are cell surface glycoproteins made up of Ig repeats. They bind in a heterophilic manner to counterreceptors on other cells and these counterreceptors are usually of the β2-integrin type [43]. *Cadherins* homophilically bind to cadherins on other cells [35]. Their short cytoplasmic tail contacts the cytoskeleton via a group of proteins called catenins, and this feature is critically involved in cadherin function. The *selectins* are heavily glycosilated cell surface proteins that bind to counterreceptors on other cells but the exact nature of such counterstructures is still largely unknown [4]. Finally, a member of the cartilage-link proteins, *CD44*, is a cell adhesion molecule involved in adhesion to hyaluronate (HA) and it is thought to act as a homing receptor on lymphocytes [22].

Integrin-mediated adhesion to ECM components and E-cadherin mediated binding to keratinocytes has been reported for melanocytes in vitro [36,44]. In the present study we investigated the adhesive characteristics of melanocytes cultured in the presence or absence of the mitogen phorbol 12-myristate 13-acetate (PMA).

MATERIALS AND METHODS

Materials and antibodies

Laminin (Ln) and collagen type IV (CoIV), both isolated from Englebreth-Holm-

Swarm mouse sarcoma cells, were purchased from Life Technologies (Grand Island, NY). Collagen type I (CoI) was isolated from rat tail. Fibronectin (Fn), isolated from human plasma, and hyaluronate (HA), isolated from human umbilical cord, were purchased from Sigma (St Louis, MO). The antibodies used are listed in Table 1.

Table 1. Monoclonal antibodies.

antibody	*antigen*	*source and reference*
NKI-beteb	gp100	Dr. Figdor, Nijmegen, The Netherlands [2]
TS2/7	α1	T-Cell Science, Cambridge MA [14]
5E8	α2	Dr. Bankert, Buffalo, NY [45]
P1B5	α3	Life Technologies, Gaithersburg, MD [41]
HP2/1	α4	Dr. Sanchez-Madrid, Madrid, Spain [30]
NKI-SAM1	α5	Dr. Figdor, Nijmegen, The Netherlands [39]
GoH3	α6	Dr. Sonnenberg, Amsterdam, The Netherlands [34]
13C2	αv	Dr. Horton, London, UK [8]
10E5	αIIb	Dr. Coller, Stoneybrook, NY [5]
4B4	β1	Coulter, Hialeah, FL [26]
CLB-LFA1/1	β2	Dr. Figdor, Nijmegen, The Netherlands [19]
23C6	αvβ3	Dr. Horton, London, UK [8]
3E1	β4	Telios, San Diego, CA [15]
P1F6	αvβ5	Life Technologies, Gaithersburg, MD [42]
SN1	β8	Dr. Nishimura, San Francisco, CA [27]
NKI-P2	CD44	Dr. Figdor, Nijmegen, The Netherlands [28]
CL203	ICAM-1	Dr. Ferrone, Valhalla, NY [24]
CBRIC2/2	ICAM-2	Dr. Springer, Boston, MA [9]
CBRIC3/2	ICAM-3	Dr. Springer, Boston, MA [10]
E1/6	VCAM-1	Dr. Bevilacqua, La Jolla, CA [29]
H18/7	E-selectin	Dr. Bevilacqua, La Jolla, CA [29]
HECD-1	E-cadherin	Takara Biomedicals, Otsu, Japan [33]
WT31	CD3	Dr. Tax, Nijmegen, The Netherlands [37]

Isolation and culturing of melanocytes and keratinocytes

Melanocytes were isolated from human foreskin epidermal cell suspensions by selective adherence and growth in HAM's F10 medium (Flow, Irvine, UK) supplemented

with 2% Ultroser-G synthetic serum (Life Technologies), glutamate, penicillin and streptomycin, 100 μM IBMX (Sigma) and 16 nM PMA (Sigma) and were cultured for a maximum of 6 passages. For some experiments, melanocytes were allowed to differentiate by removal of PMA from the medium. Freshly isolated human keratinocytes were a kind gift from Mieke Latijnhouwers (Dept. of Dermatology, University Hospital, Nijmegen, The Netherlands). Keratinocytes were cultured for a maximum of 3 passages in serum free keratinocyte growth medium containing 0.09 mM calcium and supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml recombinant epidermal growth factor (Life Technologies). Under these conditions keratinocytes proliferate and express a basal phenotype without stratification [36].

Flow cytometry

Cultured cells were harvested by short trypsinization of subconfluent monolayers and suspended in complete HAM's F10 medium. After washing with PBS containing 0.5% BSA and 0.02% azide, they were incubated with mAbs (Table 1) in PBS/BSA/azide for 30 min at 4°C. After washing with PBS/BSA/azide, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) or, in the case of GoH3 mAbs, with FITC-conjugated rabbit anti-rat Ig antibodies (Dako). Analyses were performed on an Epics Elite Flowcytometer (Coulter, Mijdrecht, The Netherlands).

Immunoprecipitation

Subconfluent monolayer cell cultures (75 cm^2) were labeled overnight at 37°C with 0.5 mCi [^{35}S]-methionine (Amersham, Houten, The Netherlands) in methionine-free medium (Flow) containing 10% dialyzed FCS. The cells were washed 2 times with PBS and incubated with NP40 lysis buffer (0.5% NP40, 0.015 M NaCl, 0.01 M Tris pH 7.5, 1.0 mM phenyl-methyl-sulphonyl-fluoride and 4 μg/ml Aprotinin) at 4°C for 10 min. Subsequently the cells were scraped off the culture flask, repeatedly aspired into syringes and forced through needles with decreasing diameter. Glycoproteins were isolated from NP40 solubilized cell extracts by adsorption to concanavalin A (Con A)-Sepharose (Pharmacia Inc., Uppsala, Sweden). Immunoprecipitations were performed as described before [20]. To compare the amount of glycoproteins in the various cell lines, equal numbers of counts of the Con A-bound fractions were used for immunoprecipitation.

Cell adhesion assay

Adhesion assays were performed as described before [7]. For adhesion to ECM components, polystyrene microtiter plates (96 flatbottomed wells; Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with 20 μg/ml of the appropriate ECM component, washed and blocked with PBS/BSA. Coupling of HA to the wells was done as described before [40]. For adhesion assays to keratinocytes, these were allowed to

adhere and spread in wells for 3 h in keratinocyte growth medium and unattached cells were washed away. Subsequently, ^{51}Cr-labeled melanocytes in DMEM (Flow) containing 0.25% BSA were seeded into the wells (10,000 cells/well) and allowed to attach for 30 min at 37°C. After washing, the adherent cells were lysed in Triton-X-100 and radioactivity was measured in a gamma-counter. For adhesion inhibition assays, melanocytes were incubated with mAbs for 30 min at 4°C before being added to the wells, and keratinocytes were incubated with mAbs for 30 min at 37°C prior to addition of labeled melanocytes.

RESULTS

Surface expression of adhesion molecules on cultured melanocytes

Of the integrin family, proliferating melanocytes in the presence of PMA expressed predominantly integrins $\alpha3\beta1$ and $\alpha v\beta3$ (Table 2). Moderate levels of $\alpha5\beta1$ and $\alpha v\beta5$ were expressed. Low expression was observed of $\alpha2\beta1$, $\alpha6\beta1$ and $\alpha v\beta8$, whereas $\alpha1\beta1$, $\alpha4\beta1$, $\alpha IIb\beta3$ and $\beta2$- and $\beta4$-integrins were absent. Of the Ig superfamily only ICAM-1 was expressed whereas ICAM-2, ICAM-3 and VCAM-1 were absent. No expression of E-selectin was detected whereas high expression of E-cadherin was observed and CD44 was very strongly expressed.

Table 2. Surface expression of adhesion molecules on melanocytes cultured in the presence of PMA.

control[1]	E-cadherin	VCAM-1	E-selectin	ICAM-1	ICAM-2
5/6[2]	85/98	7/8	4/6	41/30	5/9
ICAM-3	CD44	α1	α2	α3	α4
6/6	860/801	6/9	12/17	80/101	5/5
α5	α6	αIIb	αv	β1	β2
24/27	13/14	7/5	50/37	96/127	6/8
αvβ3	β4	αvβ5	β8		
48/32	5/5	20/22	12/12		

[1]WT31 anti-CD3 mAbs

[2]Mean fluorescence given for 2 patients.

Removal of PMA from the medium induced a shift in morphology from a dendritic bipolar morphology to a more spreaded appearance (Fig 1), it strongly increased melanin synthesis (not shown) and it arrested proliferation. In the absence of PMA, surface expression of $\alpha3\beta1$ and $\alpha5\beta1$ decreased (Fig 2). In contrast, faint expression of $\alpha4\beta1$

became detectable and expression of $\alpha 6\beta 1$ was enhanced. Similarly, for melanocytes of 1 individual, enhanced expression of $\alpha 2\beta 1$ was observed in the absence of PMA (Fig 2), but this was not found for melanocytes from 2 other individuals (not shown). No differences in expression were observed between cells cultured with or without PMA for any of the other adhesion molecules studied (not shown).

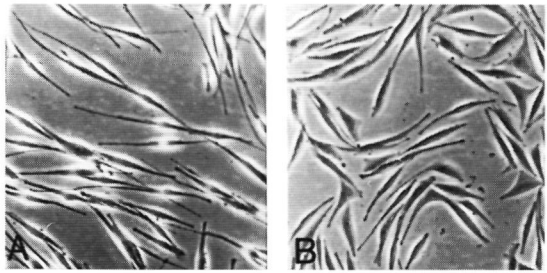


Figure 1. *Effect of PMA on the morphology of cultured melanocytes. Melanocytes were cultured in the presence (**A**) or absence (**B**) of PMA for two weeks.*

These data show that arrest of proliferation and acquirement of a differentiated phenotype of melanocytes in the absence of PMA is accompanied by selective up- and down modulation of integrin expression levels.

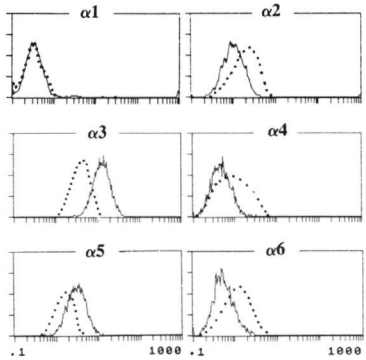


Figure 2. *Effect of PMA on the expression of integrins. Melanocytes were cultured in the presence (line) or absence (dotted line) of PMA for two weeks and incubated with anti-integrin mAbs followed by FITC-labeled second antibodies. Results are shown for one out of three individuals.*

Synthesis of integrins in cultured melanocytes

We next investigated if the shift in the level of surface expression of $\alpha 3\beta 1$ and $\alpha 6\beta 1$, upon transition from a proliferative to a diferentiated state, was reflected by changes in the level of biosynthesis of these integrins. Therefore, we immunoprecipitated the $\alpha 3$ and $\alpha 6$ chain from metabolically labeled melanocytes cultured in the presence or absence of PMA. In line with the surface expression data, synthesis of $\alpha 3$ decreased whereas synthesis of $\alpha 6$ increased in the absence of PMA (Fig 3). In melanocytes obtained from one individual we also observed induction of $\alpha 2$ synthesis in the absence of PMA but in melanocytes of another individual this was not the case (Fig 3).

These results indicate that changes in surface expression of integrins upon removal of PMA from the culture medium are accompanied by changes in the level of biosynthesis of these molecules.

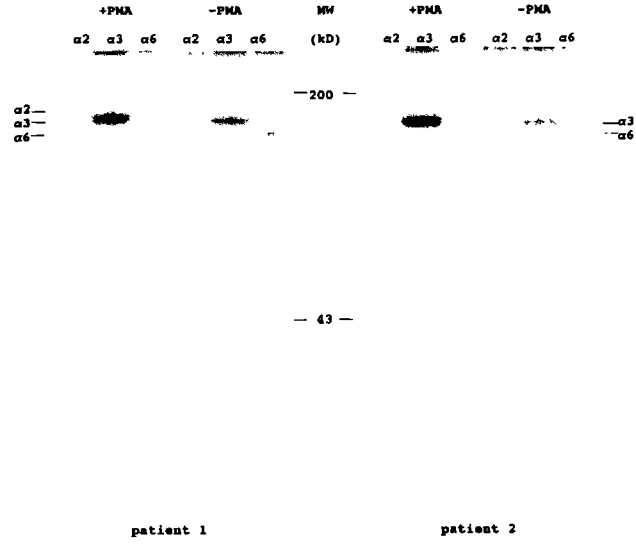


Figure 3. Effect of PMA on the biosynthesis of integrins. Melanocytes of two patients were cultured in the presence or absence of PMA for two weeks as indicated. Subconfluent monolayers were metabolically labeled overnight, lysed, and glycoproteins were isolated by adsorption to Con A-Sepharose. Equal amounts of counts from the Con A-bound fraction were used for immunoprecipitation with mAbs to α2, α3, or α6.

Effect of PMA on adhesion of melanocytes to ECM-components

As removal of PMA from the culture medium affected the expression pattern of adhesion molecules, we performed adhesion assays with melanocytes cultured in the presence or absence of PMA. Melanocytes cultured with PMA adhered to Fn and HA but they adhered very poorly to Ln and not to CoI or CoIV (Fig 4A). However, after culturing in the absence of PMA for one week, melanocytes gained the ability to adhere strongly to Ln and Co whereas adhesion to Fn and HA was unaffected. Adhesion to Fn was inhibited by mAbs to integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ in the presence (Fig 4B) or absence (not shown) of PMA. We have previously demonstrated that adhesion of melanocytes to HA is mediated by CD44 [40]. The enhanced adhesion to Ln of melanocytes grown in the absence of PMA could be blocked with anti-integrin $\beta 1$ and anti-$\alpha 6$ mAbs but not with anti-$\alpha 2$ or anti-$\alpha 3$ mAbs (Fig 4C). The induced adhesion to Co was blocked with anti-integrin $\beta 1$ and anti-$\alpha 2$ mAbs and not with anti-$\alpha 3$ or anti-$\alpha 6$ mAbs.

These results indicate that, in line with loss of expression of $\alpha 6\beta 1$ and $\alpha 2\beta 1$,

adhesion to Ln and Co through these integrins as observed for non-proliferative melanocytes, is absent from proliferating melanocytes in the presence of PMA.

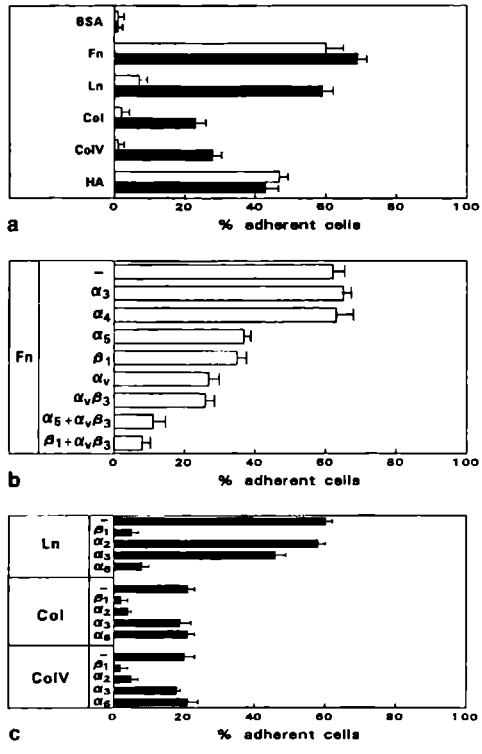


Figure 4. Effect of PMA on adhesion to ECM components. A: Melanocytes were cultured in the presence (dotted bar) or absence (filled bar) of PMA for two weeks before being tested in adhesion assays. Shown is the percentage of adherent cells to wells coated with different ECM components as indicated. B: Melanocytes cultured in the presence of PMA were allowed to adhere to Fn in the absence or presence of mAbs to integrins as indicated. C: Melanocytes cultured in the absence of PMA were allowed to adhere to wells coated with Ln, Col or ColV in the absence or presence of mAbs to integrins as indicated. For A and B one experiment of three, and for C one experiment of two is shown. Means ± s.d. from triplicate wells are shown.

Effect of PMA on adhesion of melanocytes to keratinocytes

As melanocytes expressed several receptors that have been implicated in cell-cell interactions in the epidermis (E-cadherin and integrins $\alpha2\beta1$ and $\alpha3\beta1$), we investigated adhesion to keratinocytes of melanocytes cultured in the presence or absence of PMA. Melanocytes adhered to keratinocytes under both conditions (Fig 5A). Removal of keratinocytes with EDTA and washing the wells 3 times with DMEM/BSA shortly before addition of the labeled melanocytes completely abrogated adhesion, indicating that ECM proteins produced by the keratinocytes and attached to the wells were not involved, and that indeed cell-cell interactions were studied. We used mAbs to integrin subunits $\alpha2$, $\alpha3$, and $\beta1$, and to E-cadherin to inhibit adhesion. MAbs to integrin $\beta1$ and to E-cadherin moderately inhibited adhesion to keratinocytes and the combination of these mAbs inhibited adhesion for approximately 60% (Fig 5B). No effect was observed of the mAbs to integrin $\alpha2$ or $\alpha3$ subunits.

These findings indicate that E-cadherin/$\beta1$-integrin-mediated contacts of melanocytes with proliferating non stratifying keratinocytes are not influenced by PMA.

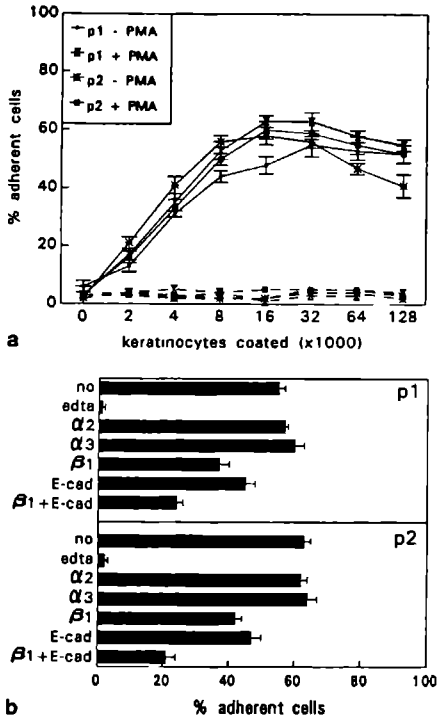


Figure 5. Effect of PMA on adhesion to keratinocytes. A: Melanocytes from two individuals were grown in the absence or presence of PMA for two weeks before being allowed to adhere to wells coated with increasing amounts of keratinocytes as indicated. In a parallel experiment, keratinocytes were removed by EDTA and wells were washed prior to addition of the labeled melanocytes (dashed lines). B: Melanocytes of two individuals were cultured in the presence of PMA and allowed to adhere to wells coated with 1.5x10⁴ keratinocytes. Melanocytes and keratinocytes were incubated with 10 μg/ml of mAbs to α2 (5E8), α3 (P1B5), β1 (4B4), E-cadherin (HECD-1) or combinations as indicated. For A one experiment of three, and for B one experiment of two is shown. Means ± s.d. from triplicate wells are shown.

DISCUSSION

In the present study, we have investigated the adhesive characteristics of cultured normal human melanocytes maintained either in a differentiated non-proliferative state (characterized by cell spreading, high level of melanin synthesis, and lack of cell division) or in a highly proliferative state induced by the phorbol ester PMA. Expression of adhesion molecules of the integrin-, Ig-, cadherin-, selectin-, and CD44 families was determined and the role of these receptors in adhesion to ECM components and to keratinocytes was explored.

The finding that melanocytes express predominantly $\alpha 3\beta 1$ of the integrins and that they have E-cadherin expression is in line with earlier reports [36,44]. We also detect expression of integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ and of ICAM-1, three adhesion molecules that have been implicated in melanocytic tumor progression [3,6,18]. This is not due to any effect of the tumor promotor PMA since these molecules can also be detected in the absence of PMA. Expression may be induced by other culture conditions since these molecules are absent from melanocytes in situ. The capacity of melanocytes to express such melanocytic tumor progression-associated adhesion molecules may be important

features during embryonic development and wound healing.

We find that proliferating melanocytes adhere strongly to Fn but not or hardly to Ln and Co. This is probably due to the very low expression of $\alpha 2\beta 1$ Ln/Co- and $\alpha 6\beta 1$ Ln receptors in the presence of PMA and to the absence of $\alpha 1\beta 1$ Ln/Co binding integrin. Even though $\alpha 3\beta 1$ is a receptor for Ln, Co, and Fn, its high level of expression does not provide melanocytes with the capacity to adhere to Ln and Co, and $\alpha 3\beta 1$ is not involved in the adhesion to Fn. It has been shown that $\alpha 3\beta 1$ can be involved in migration of melanocytes [44]. In line with the results from that study, we find that melanocyte adhesion to Fn is mediated by integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$. However, others have shown that in neonatal but not fetal melanocytes, $\alpha 3\beta 1$ can be involved in adhesion to Fn [31]. Such subtle discrepancies may possibly be explained by differences in culture conditions that may effect the activity of the adhesion molecules.

A marked effect of PMA is observed specifically on melanocyte adhesion to Ln and Co. In the absence of PMA, melanocytes adhere strongly to these basement membrane glycoproteins. Our finding that long term culturing of melanocytes in the presence of PMA abrogates adhesion to Ln and Co is in line with findings for keratinocytes [1]. However, opposite to its effect on melanocytes, PMA induces terminal differentiation of keratinocytes. Furthermore, in keratinocytes a general decrease of $\beta 1$-integrin expression is observed whereas melanocytes have a selective increase of $\alpha 3\beta 1$ and $\alpha 5\beta 1$ expression in the presence of PMA. Our immunoprecipitation data show that increased surface expression is accompanied by an increased level of synthesis. For other $\beta 1$ integrins ($\alpha 2\beta 1$, $\alpha 4\beta 1$ and $\alpha 6\beta 1$) we find the opposite effect, namely that PMA selectively downmodulates surface expression and synthesis. In addition, even though modulation of $\alpha 2\beta 1$ expression was only observed in melanocytes from one individual, in all cases we observed a strong effect of PMA on $\alpha 2\beta 1$-mediated adhesion to Co. This suggests that PMA influences the activity of $\alpha 2\beta 1$ as well. This effect is opposite to the effect observed when T-cells are incubated for a period of only a few minutes with PMA [32]. Such a treatment enhances the affinity of $\beta 1$ integrins on leukocytes, whereas long term exposure of melanocytes to PMA appears to reduce the affinity of $\alpha 2\beta 1$. Thus, PMA-induced proliferation of melanocytes selectively up- and downmodulates expression, synthesis, and activity of integrins, which leads to a loss of adhesion to basement membrane components Ln and Co.

Our finding that melanocytes can use E-cadherin for binding to keratinocytes is in line with the report from Tang et al. [36], but we find that E-cadherin and $\beta 1$-integrins are involved to approximately the same extent whereas Tang et al. report a major role for E-cadherin. Again, different culture conditions may influence the activity of the integrins. Even though $\alpha 2\beta 1$ and $\alpha 3\beta 1$ have been implicated in epidermal cell-cell interactions [21], up to 10 μg/ml of inhibitory mAbs to these integrins have no effect on melanocyte-keratinocyte interactions. This suggests that other $\beta 1$-integrins may be involved. No differences are observed between proliferative and non-proliferative melanocytes,

regarding adhesion to keratinocytes. For E-cadherin, this is in line with the finding that expression of E-cadherin is not influenced by PMA and it may suggest that expression and/or activation of the unidentified β1-integrin is not affected by PMA either.

The differential modulation by PMA of melanocyte adhesion to basement membrane components and keratinocytes, may be paralleled in vivo by effects of keratinocyte-derived cytokines [23]. One might speculate that by differentially affecting attachment to the basement membrane and to other cells, such modulation may influence proliferation and migration of melanocytes during embryogenesis, wound healing, and the initial steps of melanocytic tumor progression.

ACKNOWLEDGEMENTS

We thank Drs. Richard Bankert, Michael Bevilacqua, Barry Coller, Soldano Ferrone, Carl Figdor, Michael Horton, Stephen Nishimura, Francesco Sánchez-Madrid, Arnoud Sonnenberg, Timothy Springer, and Wil Tax for generously providing antibodies. We are indebted to Mieke Latijnhouwers for providing freshly isolated keratinocytes. This work was financially supported by the Dutch Cancer Society (Grant NUKC 91-09).

REFERENCES

1. Adams JC, Watt FM. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha 5\beta 1$ integrin loss from the cell surface. Cell 63, 425-435, 1990.

2. Adema GJ, de Boer AJ, Van 't Hullenaar R, Denijn M, Ruiter DJ, Vogel AM, Figdor CG. Melanocyte lineage specific antigens recognized by monoclonal antibodies, NKI-beteb, HMB 50, and HMB 45 are encoded by a single cDNA. Am J Pathol 143, 1579-1585, 1993.

3. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: association of the $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

4. Bevilacqua M, Butcher E, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G. Selectins: a family of adhesion receptors. Cell 67, 233, 1991.

5. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 72, 325-338, 1983.

6. Danen EHJ, ten Berge PJM, van Muijen GNP, van 't Hof Grootenboer AE, Bröcker E-B, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor expression in melanocytic tumor progression. Histopathol 24, 249-256, 1994.

7. Danen EHJ, van Muijen GNP, van de Wiel-van Kemenade E, Jansen CFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. Int J Cancer 54, 315-321, 1993.

8. Davies J, Warwick J, Totty N, Phillip R, Helfrich M, Horton M. The osteoclast functional antigen,

implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J Cell Biol 109, 1817-1826, 1989.

9. De Fougerolles AR, Springer TA. Intercellular adhesion molecule 3, a third adhesion counterreceptor for LFA-1. J Exp Med 175, 185-190, 1992.

10. De Fougerolles AR, Stacker SA, Schwarting R, Springer TA. Characterization of ICAM-2 and evidence for a third counterreceptor for LFA-1. J Exp Med 174, 253-259, 1991.

11. De Luca M, D'Anna F, Bondanza S, Franzi AT, Cancedda R. Human epithelial cells induce melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis. J Cell Biol 107, 1919-1926, 1988.

12. Fitzpatrick TB, Szabo G, Seiji M, Quvedo WCJr. Biology of the melanin pigmentary system. In: TB Fitzpatrick, AZ Eisen, K Wolff, IM Freedberg, KF Ansten (eds) Dermatology in general medicine, pp 131-167. McGraw-Hill, New York, 1979.

13. Gilchrest BA, Albert LS, Karasik RL, Yaar M. Substrate influences human epidermal melanocyte attachment and spreading in vitro. In vitro Cell Dev Biol 21, 114-120, 1985.

14. Hemler ME, Sánchez-Madrid F, Flotte TJ, Krensky AM, Burakoff SJ, Bhan AK, Springer TA. Glycoproteins of 210,000 and 130,000 m w. on activated T-cells: Cell distribution and antigenic relation to components on resting cells and T-cell lines. J Immunol 132, 3011-3018, 1984.

15. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Basement membrane diversity detected by monoclonal antibodies. Differentiation 26, 49-54, 1984.

16. Hynes RO Integrins versatility, modulation, and signalling in cell adhesion Cell 68, 11-25, 1992.

17. Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. Cell 68, 303-322, 1992.

18. Johnson JP, Stade BG, Holzmann B, Schwäble P, Riethmüller G. De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc Natl Acad Sci USA 86, 641-644, 1989

19. Keizer GD, Visser W, Vliem M, Figdor CG. A monoclonal antibody (NKI-L16) directed against a unique epitope on the alpha chain of LFA-1 induces homotypic cell-cell interaction. J Immunol 140, 1393-1400, 1988.

20. Klein CE. A transformation associated 130 KD cell surface glycoprotein is growth controlled in normal human cells. J Exp Med 167, 1684-1696, 1988.

21. Larjava H, Peltonen J, Akiyama SK, Yamada SS, Gralnicck HR, Uitto J, Yamada KM. Novel function of $\beta 1$ integrins in keratinocyte cell-cell adhesion. J Cell Biol 110, 803-815, 1990.

22. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. Adv Immunol 54, 271-335, 1994.

23. Luger TA, Schwarz T. Evidence for an epidermal cytokine network. J Invest Dermatol 95, 100S-104S, 1990.

24. Maio M, Tessitori G, Pinto A, Temponi M, Colombatti A, Ferrone S. Differential role of distinct determinants of intercellular adhesion molecule-1 in immunologic phenomena. J Immunol 143, 181-188, 1989.

25. Moretti S, Martini L, Berti E, Pinzi C, Gianotti B. Adhesion molecule profile and malignancy of melanocytic lesions. Melanoma Res 3, 235-239, 1993.

26. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985

27. Nishimura SL, Sheppard D, Pytela R. Integrin $\alpha v \beta 8$· interaction with vitronectin and functional divergence of the $\beta 8$ cytoplasmic domain. J Biol Chem 269, 28708-28715, 1994.

28. Pals ST, Hogervorst F, Keizer GD, Thepen T, Horst E, Figdor CG Identification of a widely

distributed 90 kDa-glycoprotein that is homologous to the hermes-1 human lymphocyte homing receptor. J Immunol 143, 851-857, 1989.

29. Rice GE, Bevilacqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246, 1303-1306, 1989.

30. Sánchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-3: a novel polypeptide association within the VLA molecular complex: Cell distribution and biochemical characterization. Eur J Immunol 16, 1343-1349, 1986.

31. Scott G, Ryan DH, McCarthy JB. Molecular mechanisms of human melanocyte attachment to fibronectin. J Invest Dermatol 99, 787-794, 1992.

32. Shimizu Y, van Seventer G, Horgan KJ, Shaw S. Regulated expression and binding of three VLA (β1) integrin receptors on T cells Nature 345, 250-253, 1990.

33. Shimoyama Y, Hirohashi S, Hirano S, Noguchi M, Shimosato Y, Takeichi M, Abe O. Cadherin cell adhesion molecules in human epithelial tissue and carcinomas. Cancer Res 49, 2128-2133, 1989.

34. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. J Biol Chem 264, 13745-13750, 1987.

35. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion Annu Rev Biochem 59, 237-252, 1990.

36. Tang A, Eller MS, Hara M, Yaar M, Hirohashi S, Gilchrest BA. E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci 107, 983-992, 1994.

37. Tax WJM, Willems HW, Reekers PPM, Capel PJA, Koene RAP. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 304, 445-447, 1982.

38. Valyi-Nagy IT, Hirka G, Jensen PJ, Shih I-M, Juhasz I, Herlyn M. Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. Lab Invest 69, 152-159, 1993.

39. Van de Wiel-van Kemenade E, Van Kooyk Y, De Boer AJ, Huijbens SRJF, Weder P, Van de Kasteele W, Melief CJF, Figdor CG Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

40. Van Muijen GNP, Danen EHJ, Veerkamp J, Ruiter DJ, Lesley J, Van den Heuvel LPWJ Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity. Int J Cancer 61, 241-248, 1995.

41. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits J Cell Biol 105, 1873-1884, 1987.

42. Wayner EA, Orlando RA, Cheresh DA. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J Cell Biol 113, 919-929, 1991.

43. Williams AF, Barclay AN. The immunoglobulin superfamily: domains for cell surface recognition Annu Rev Immunol 6, 381-405, 1988.

44. Zambruno G, Marcisio PC, Melchiori A, Bondanza S, Cancedda R, De Luca M. Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes J Cell Sci 105, 179-190, 1993.

45. Zijlstra S, Chen FA, Ghosh SK, Repasky EA, Rao U, Takita H, Bankert RB. Membrane associated glycoprotein (gp160) identified on human lung tumor by a monoclonal antibody. Cancer Res 48, 2768-2773, 1986.

Chapter 3

E-cadherin expression in human melanoma

Melanoma Res, in press

E-cadherin expression in human melanoma

Erik HJ Danen[1], Teunis J de Vries[1], Renato Morandini[2], Ghanem G Ghanem[2], Dirk J Ruiter[1], and Goos NP van Muijen[1]

[1]*Department of Pathology, University Hospital, Nijmegen, The Netherlands, and*

[2]*Laboratory of Oncology and Experimental Surgery, L.O.C.E., J. Bordet Institute, University of Brussels, Belgium*

Loss of expression of E-cadherin, the major cell-cell adhesion receptor on keratinocytes, has been linked to tumor progression in various carcinomas. As E-cadherin has been reported to be expressed in cultured human melanocytes, we questioned whether loss of E-cadherin expression may also be related to melanocytic tumor progression. Flowcytometrical analysis demonstrated that E-cadherin was expressed on cultured normal melanocytes and nevus cells. Two non-invasive/non-metastatic melanoma cell lines showed low expression and 4 invasive/metastatic melanoma cell lines did not express E-cadherin. Immunohistochemistry on frozen sections of human melanocytic lesions, resulted in diffuse staining of 1/23 common nevocellular nevi and 1/13 dysplastic nevi, and no staining was observed in 7/7 early primary melanomas (≤ 1.5 mm). Staining was observed in 4/20 advanced primary melanomas (> 1.5 mm) and 5/35 melanoma metastases. Thus, even though E-cadherin is expressed in cultured melanocytes and nevus cells and lost in invasive/metastatic melanoma cells in vitro, it is rarely found in early stages of melanocytic tumor progression in situ, and surprisingly, some expression can be observed in 10-20% of lesions of advanced stages.

INTRODUCTION

E-cadherin is a calcium-dependent epithelial cell adhesion receptor that is clustered in adherens junctions. In these regions, E-cadherin molecules on opposing cells bind to each other [6]. In addition to these homophilic interactions, E-cadherin can heterophilically bind integrin $\alpha e\beta 7$ on lymphocytes [8]. Binding of a complex of proteins called catenins to its cytoplasmic domain is crucial for the adhesive function of E-cadherin [28].

Reduction of intercellular adhesiveness is a prerequisite for the invasive behavior of carcinomas, and the breakdown of the adherens junctions is central in this process [29]. Indeed, antibodies to E-cadherin can induce invasiveness of otherwise non-invasive epithelial cells [3], and conversely, E-cadherin cDNA transfection into highly invasive, E-cadherin negative carcinoma cells, greatly reduces their invasive capacity [37]. Furthermore, loss of (membrane-associated) E-cadherin expression was found to be correlated with lymph node metastasis of squamous cell carcinoma [25], with dedifferentiation of meningiomas [31], with high Gleason grade of prostate carcinomas [32], with infiltrative growth of basal cell carcinoma [24], with dedifferentiation and metastasis of breast carcinoma [15,20,21], with dedifferentiation, high Dukes stage, and metastasis of colon carcinoma [13,18], with poor prognosis of bladder cancer (in combination with gp78) [22], with dedifferentiation of thyroid carcinoma [7], and with lymph node metastasis, high grade, and advanced stage of pancreatic carcinoma [23].

Recently, it has been reported that E-cadherin is expressed on cultured melanocytes where it mediates adhesion to keratinocytes [30]. Therefore, in the present study, we investigated the hypothesis that loss of E-cadherin might also be related to melanocytic tumor progression.

MATERIALS AND METHODS

Cell lines and culture conditions

The cutaneous melanoma cell lines included IF6 and 530 (non-metastatic in nude mice and non-invasive through a human amniotic basement membrane), and M14, Mel57, BLM, and MV3 (metastatic and invasive) [9,34,35]. Uveal melanoma cell lines included Mel202 [19], provided by Dr. BR Ksander, Boston, MA; 92-I [11], provided by Dr. I de Waard-Siebinga, University Hospital, Leiden, The Netherlands; and OCM-1 [17], provided by Dr. GPM Luyten, Rotterdam, The Netherlands. All melanoma cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Flow laboratories, Irvine, UK) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Isolation and propagation of human foreskin melanocytes were performed as previously described [27]. Nevus cells were isolated from giant congenital nevi. Melanocytes and nevus cells were cultured for a maximum of 5 passages in Ham's F10

(Flow laboratories) supplemented with 2% Ultroser-G synthetic serum (Gibco, Grand Island, NY), glutamate, penicillin, streptomycin, 0.1 mM IBMX (Sigma, St Louis, MO) and 16 nM phorbol 12-myristate 13-acetate (PMA) (Sigma).

Flowcytometry

Cells were harvested by short trypsinization of subconfluent monolayers, suspended in complete culture medium, and washed with phosphate buffered saline (PBS) containing 0.5% BSA and 0.02% azide, and 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ to preserve E-cadherin integrity. Subsequently cells were incubated with 6F9 anti-E-cadherin mAb [14] (Eurodiagnostica, Apeldoorn, The Netherlands) in PBS/BSA/azide/Ca^{2+}/Mg^{2+} for 30 min at 4°C. After washing, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). Analyses were performed on an Epics Elite flowcytometer (Coulter, Mijdrecht, The Netherlands).

Lesions

Lesions were obtained from patients at the University Hospital, Nijmegen, The Netherlands or provided by Dr. E-B Bröcker, Würzburg, Germany. Based on histopathologic examination of paraffin sections, lesions were divided into five classes: common nevocellular nevus (NN) (n=23), dysplastic [12] (atypical) nevus (DN) (n=13), early primary melanoma (tumor thickness$\leq$1.5 mm; ePM) (n=7), advanced primary melanoma (tumor thickness>1.5 mm; aPM) (n=20), and melanoma metastasis (MM) (n=35). Representative samples were snap frozen in liquid nitrogen and stored at −80°C until sectioning.

Immunohistochemistry

Four μm frozen sections were fixed in 3% paraformaldehyde for 20 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes and subsequently incubated at room temperature with 6F9 E-cadherin mAb for 1 h. All buffers contained 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ to preserve E-cadherin integrity. After washing with PBS, bound mAbs were visualized using the peroxidase-based Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole as substrate. After counterstaining with Meyers hematoxylin sections were mounted with Kaisers glycerin/gelatin (Merck, Darmstadt, Germany).

Melanocytic cells were identified in parallel HE-stained sections and by staining frozen sections with NKI-beteb anti-gp100 mAb [2].

To investigate if E-cadherin expression is inversely related to melanocytic tumor progression, we first analyzed expression on cultured human melanocytes isolated from 2 individuals, on cultured nevus cells isolated from 2 individuals, and on a panel of 6 human cutaneous melanoma cell lines with different invasive and metastatic potential. Both melanocyte cultures stained positive with the 6F9 E-cadherin mAb (Fig 1). The 2 nevus cell cultures expressed E-cadherin as well, though the level of expression was somewhat lower than that found on melanocytes. Culturing melanocytes or nevus cells in the absence of PMA for 1 week, did not affect E-cadherin expression (Fig 1). Of the panel of cutaneous melanoma cell lines, only the non-invasive/non-metastatic cell lines IF6 and 530, expressed a very low amount of E-cadherin, whereas the invasive and metastatic cell lines M14, Mel57, BLM, and MV3 were negative (Fig 1). In addition, no E-cadherin could be detected on 3 uveal melanoma cell lines (Mel202, 92-I, and OCM-1) (not shown).

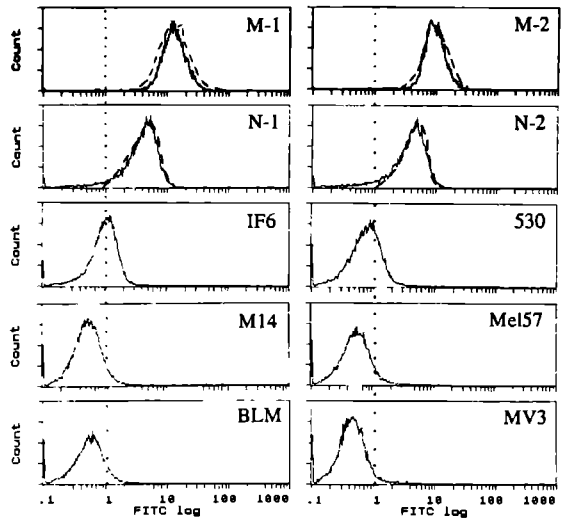


Figure 1. *E-cadherin expression on cultured human melanocytic cells. Melanocytes (M), nevus cells (N), and cutaneous melanoma cell lines were stained with 6F9 E-cadherin mAb. Dashed graphs represent melanocytes and nevus cells cultured in the absence of PMA for 1 week. The dotted vertical line indicates the gate set with control Ig. One representative experiment of 3 is shown.*

We next investigated E-cadherin expression on frozen sections of lesions from different stages of human melanocytic tumor progression. Epidermal keratinocytes, in all lesions that contained epidermis, stained strongly showing a membranous aspect. Because of the close contact of epidermal melanocytes with keratinocytes it was not possible to determine if melanocytes in normal skin expressed E-cadherin. We observed some diffuse staining with 6F9 mAb of melanocytic cells only in 1/23 NN (Fig 2a,b,c) and 1/13 DN. All 7 ePM tested lacked E-cadherin expression whereas 4/20 aPM and 5/35 MM did express E-cadherin. Heterogenous expression was observed in those lesions; some cells with membranous expression, some with cytoplasmic staining, and some being negative (Fig 2d,e,f). More than 50% of tumor cells stained in all positive lesions. The positive MM originated from liver, kidney, and lymph nodes. As E-cadherin can mediate binding of lymphocytes, we checked for enhanced lymphocyte infiltration in the positive aPM and MM lesions but no clusters of immune cells were present.

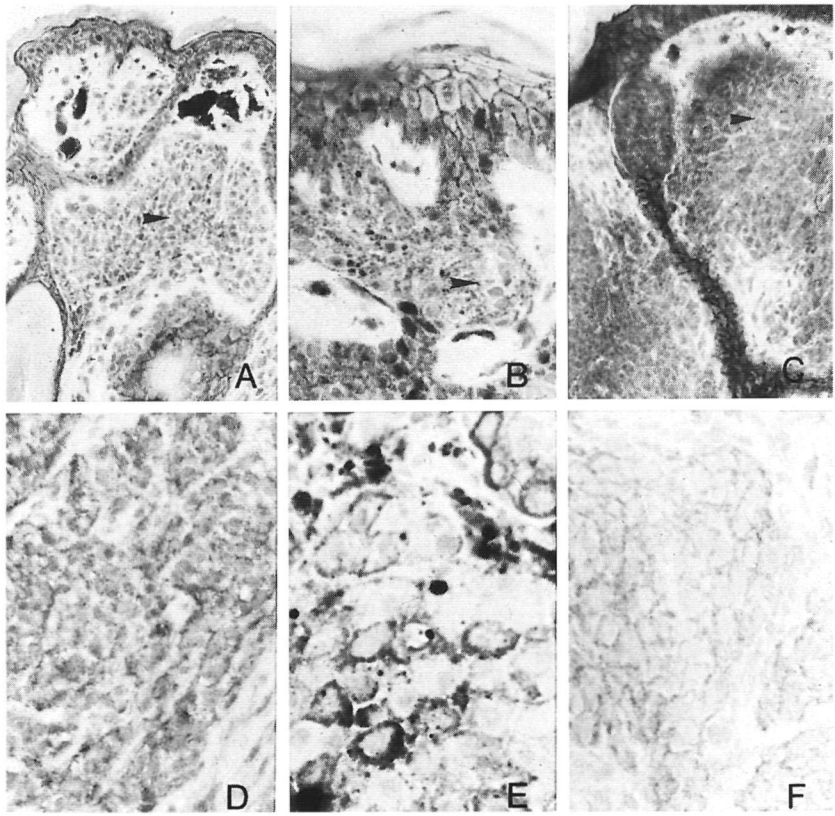


Figure 2. *Immunohistochemical E-cadherin staining in human melanocytic lesions. Frozen sections of common nevocellular nevi* ***(A,B,C)*** *and melanoma metastases* ***(D,E,F)*** *were stained with 6F9 mAb. Note that in* ***A***, ***B***, *and* ***C*** *keratinocytes stain but only nevus cells (arrows) in* ***C*** *stain diffusely.*

Changes of adhesive properties are thought to play an important role in the process of tumor progression. Not only do metastatic tumor cells have to gain the capacity to adhere to basement membranes and to the extracellular matrix in the target organ, but loss of homotypic cell-cell adhesion is important for the initial invasion into surrounding tissues [4,16,36]. For invasive carcinomas, downmodulation of intercellular adhesion involves the breakdown of adherens junctions [29]. The cell-cell adhesion receptor E-cadherin is clustered in these structures on keratinocytes and its (membrane-associated) expression is lost with tumor progression in carcinomas [5]. E-cadherin is also expressed on cultured melanocytes where it can mediate adhesion to keratinocytes [30]. As keratinocytes control proliferation and differentiation of melanocytes [10,33], we hypothesized that loss of E-cadherin may also occur in melanocytic tumor progression.

Our finding that E-cadherin is expressed on cultured melanocytes and nevus cells and that it is lost on invasive and metastatic melanoma cell lines, confirms and extends the findings from an earlier report where expression was reported on cultured melanocytes [30]. Thus, in vitro, loss of E-cadherin seems to be related to melanocytic tumor progression. It is interesting in this respect that the 2 melanoma cell lines that are non-invasive and non-metastatic [9,34] have some weak remaining expression.

To the best of our knowledge, no studies have been published on E-cadherin expression on melanocytic cells in situ. Based on the in vitro findings it might be expected that melanocytes and nevi would express E-cadherin. However, we do not find expression in 22/23 NN and 12/13 DN. It is not possible to determine expression on normal melanocytes in situ since these cells are in close contact with surrounding keratinocytes which express high levels of E-cadherin at their surface. The fact that almost all NN and DN are negative whereas nevus cell cultures express E-cadherin, suggests that culturing the nevus cells induces expression. In analogy, the strong surface expression observed on cultured normal melanocytes may be a culture artefact as well. In this study, the melanocytes and nevus cells are cultured both in the presence and in the absence of the phorbol ester PMA, an agent that influences expression and function of another group of adhesion molecules called integrins [1,26]. We observe no effect of PMA on E-cadherin expression on these cell types. Possibly, other factors present in the culture medium may play a role.

In contrast to the hypothesis that E-cadherin may be lost with melanocytic tumor progression, expression is observed in 10-20% of the aPM and MM. Melanoma cells may produce a transcription factor that induces abberant E-cadherin expression, but the heterogenous expression observed in these lesions (cells with some membranous staining and others negative) does not suggest any functional consequences for the adhesive behavior of the cells. Whether E-cadherin-mediated homotypic aggregation can enhance melanoma metastasis, i.e. by prolonging survival of melanoma cells in the blood stream,

is not known. As E-cadherin can also bind integrin $\alpha e\beta 7$ on T-cells [8], its expression on melanoma cells may facilitate lymphocyte infiltration of the tumor. However, the E-cadherin positive melanomas do not show increased numbers of tumor infiltrating lymphocytes compared to E-cadherin negative melanomas, suggesting that this interaction does not occur in the lesions tested.

In conclusion, we find that loss of E-cadherin correlates with increased malignancy of cultured melanocytic cells, whereas E-cadherin is hardly detectable in early stages of melanocytic tumor progression in situ and emerges in a minor portion of advanced primary melanomas and melanoma metastases.

ACKNOWLEDGEMENTS

We thank Drs. BR Ksander, GPM Luyten, and I de Waard-Siebinga for providing the uveal melanoma cell lines, Dr. EB Bröcker for providing some of the lesions, and Mrs. TW Aalders for helpfull advice on the immunohistochemistry procedure. This work was financially supported by the Dutch Cancer Society (Grant NUKC 91-09).

REFERENCES

1. Adams JC, Watt F. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha 5\beta 1$ integrin loss from the cell surface. Cell 63, 425-435, 1990.

2. Adema GJ, De Boer AJ, Van 't Hullenaar R, Denijn, M, Ruiter DJ, Vogel AM, Figdor CG. Melanocyte lineage specific antigens recognized by monoclonal antibodies NKI-beteb, HMB50, and HMB45 are encoded by a single cDNA. Am J Pathol, 143, 1597-1585, 1993.

3. Behrens J, Mareel MM, Van Roy FM, Birchmeier W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 108, 2435-2447, 1989.

4. Birchmeier W. E-cadherin as a tumor (invasion) suppressor gene. BioEssays 17, 97-99, 1995.

5. Birchmeier W, Hulsken J, Behrens J. Adherens junction proteins in tumor progression Cancer Surveys 24, 129-140, 1995.

6. Boller K, Vestweber D, Kemler R. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J Cell Biol 100, 327-332, 1985.

7. Brabant C, Hoang-VU C, Cetin Y, Dralle H, Scheumann G, Molne J, Hansson G, Jansson S, Ericson LE, Nilsson M. E-cadherin: a differentiation marker in thyroid malignancies. Cancer Res 63, 4982-4993, 1994.

8. Cepec KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin Nature 372, 190-193, 1994.

9. Danen EHJ, Ruiter DJ, van Muijen GNP. Mechanisms of melanoma cell adhesion to fibronectin. Biochem Soc Transactions, 23, 403S, 1995.

10. De Luca M, D'Anna F, Bondanza S, Franzi AT, Cancedda R. Human epithelial cells induce

melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis. J Cell Biol 107, 1919-1926, 1988.

11. De Waard-Siebinga I, Blom D-JR, Griffioen M, Schrier PI, Hoogendoorn E, Beverstock G, Danen EHJ, Jager MJ. Establishment and characterization of a uveal melanoma cell line. Int J Cancer 62,155-166, 1995.

12. De Wit PEJ, Van 't Hof-Grootenboer B, Ruiter DJ, Bondi R, Bröcker EB, Cesarini JP, Hastrup N, Hou-Jensen K, Mackie RM, Scheffer E, Suter L, Urso C. Validity of the histopathological criteria used for diagnosing dysplastic naevi. Eur J Cancer, 29A, 831-839, 1993.

13. Dorodi S, Sheffield JP, Poulsom R, Northover JMA, Hart IA. E-cadherin expression in colorectal cancer: an immunohistochemical and in situ hybridization study. Am J Pathol 142, 981-986, 1993.

14. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeier W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 113, 173-185, 1991.

15. Gamallo C, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol 142, 987-993, 1993.

16. Hart IR, Birch M, Marshall JF. Cell adhesion receptor expression during melanoma progression and metastasis. Cancer Metast Rev 10, 115-128, 1991.

17. Kan-Mitchell J, Mitchell MS, Rao N, Liggett PE. Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. Invest Ophthalmol Vis Sci 30, 829-834, 1989.

18. Kinsela AR, Green B, Lepts GC, Hill CL, Bowie G, Taylor BA The role of cell adhesion molecule E-cadherin in large bowel tumor cell invasion and metastasis. Brit J Cancer 67, 904-909, 1993.

19. Ksander BR, Rubsamen BE, Olsenk R, Cousius SW, Streilein JW. Studies of tumor-infiltrating lymphocytes from a human choroidal melanoma. Invest Ophthalmol Vis Sci 32, 3198-3208, 1991.

20. Moll R, Mitze M, Frixen UH, Birchmeier W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. Am J Pathol 143, 1731-1742, 1993.

21. Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayasho T, Takatsuka Y, Matsuyoshi N, Hirano S, Takeichi M. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res 53, 1696-1701, 1993.

22. Otto T, Birchmeier W, Schmidt U, Hinke A, Schipper J, Rubben H, Raz A. Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas. Cancer Res 54, 3120-3123, 1994.

23. Pignatelli M, Ansari TW, Gunter P, Liu D, Hirano S, Takeichi M, Kloppel G, Lemoine NR. Loss of membranous E-cadherin expression in pancreatic cancer: correlation with lymph node metastasis, high grade, and advanced stage. J Pathol 174, 243-248, 1994.

24. Pizarro A, Benito N, Navarro P, Palacios J, Cano A, Quintanilla M, Contreras F, Gamalo C. E-cadherin expression in basal cell carcinomas Brit J Cancer 69, 157-162, 1993.

25. Schipper JH, Frixen UH, Behrens J, Unger A, Jahnke K, Birchmeier W. E-cadherin expression in squamous cell carcinoma of head and neck inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res 51, 6328-6337, 1991.

26. Shimizu Y, van Seventer G, Horgan KJ, Shaw S. Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature 345, 250-253, 1990.

27. Smit NPM, Westerhof W, Asghar SS, Pavel S, Siddiqui AH. Large scale cultivation of human melanocytes using collagen-coated sephadex beads (cytodex 3). J Invest Dermatol 92, 18-21, 1989.

28. Takeichi M. Cadherin cell adhesion receptors as a morphogenic regulator Science 251, 14551-14555, 1991

29. Takeichi M. Cadherins in cancer· implications for invasion and metastasis. Curr Opin Cell Biol 5, 806-811, 1993.

30. Tang A, Eller MS, Hara M, Yaar M, Hirohashi S, Gilchrest BA E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci 107, 983-992, 1994.

31. Tohma Y, Yamashima T, Yamashita J. Immunohistochemical localization of cell adhesion molecule E-cadherin in human arachnoid villi and meningiomas. Cancer Res 52, 1981-1987, 1992

32. Umbas R, Schalken J, Aalders TW, Carter BS, Karthaus HF, Schaafsma HE, Debruyne FM, Isaacs WB. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high grade prostate cancer. Cancer Res 52, 5104-5109, 1992.

33. Valgyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhash I, Herlyn M. Undifferentiated keratinocytes control growth, morphology, and antigen-expression of normal melanocytes through cell-cell contacts Lab Invest 69, 152-159, 1993.

34. Van Muijen GNP, Cornelissen IMHA, Jansen CFJ, Figdor CG, Johnson JP, Bröcker EB, Ruiter DJ. Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin Expl Metast, 9, 259-272, 1991.

35. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. Int J Cancer 48, 85-91, 1991.

36. Van Roy F, Mareel M. Tumour invasion: effects of cell adhesion and motility. Trends Cell Biol 2, 163-169, 1992.

37. Vleminckx K, Vakaet L, Mareel Jr M, Fiers W, Van Roy F. Genetic manipulation of cadherin expression by epithelial tumor cells reveals an invasion suppressor role Cell 66, 107-119, 1991

Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor expression in melanocytic tumor progression

Histopathol **24**, 249-256, 1994

Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor expression in melanocytic tumor progression

Erik HJ Danen[1], Paul JM ten Berge[1], Goos NP van Muijen[1], Anna E van 't Hof Grootenboer[1], Eva B Bröcker[2], and Dirk J Ruiter[1]

[1]*Department of Pathology, University Hospital Nijmegen, The Netherlands, and*

[2]*Department of Dermatology, University Hospital Würzburg, Germany*

Cell adhesion is crucial in the process of tumor progression. As integrins are important receptor molecules involved in cell adhesion, we studied the distribution of the α1-6, αv, αIIb, β1, β3, and β4 integrin subunits in tissue sections of common nevocellular nevi (n=22), dysplastic nevi (n=16), thin- (n=24) and thick primary cutaneous melanomas (n=28), and melanoma metastases (n=25). Decrease of α6 and β4, and increase of α4 and αv were found to be correlated with melanocytic tumor progression. Furthermore, expression of α5 and β3 was only detected in primary melanoma and melanoma metastasis. Our findings indicate that during melanocytic tumor progression alterations in integrin expression occur, the most striking being emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor.

INTRODUCTION

Tumor progression can be considered to be composed of a series of discrete sequential steps leading to metastasis [29]. It may be expected that molecules that mediate homotypic or heterotypic interactions between cells or interactions between cells and their extracellular surrounding are involved in several of these steps [23]. Loss of adhesive properties for the normal location but also requirement of adhesive properties for foreign matrices and other cells may be important characteristics of malignant tumor cells.

The last few years, a group of cell surface receptors, called integrins, has been extensively studied and characterized as summarized in several reviews [14,15,32]. Integrins are α-β heterodimeric transmembrane glycoproteins and subsets can be formed on the basis of a common β-subunit. So far, a β1, a β2, and a β3 subfamily have been described, and at least 5 additional β-subunits have been discovered. Integrins mediate cell-cell and cell-extracellular matrix (ECM) interactions and the involvement of some of these or related molecules in tumor progression has been demonstrated. Thus, it has been shown that a) the VLA-5 fibronectin receptor (integrin $\alpha 5\beta 1$) can suppress the transformed phenotype of Chinese hamster ovary (CHO) cells [11], b) the laminin and collagen receptor VLA-2 (integrin $\alpha 2\beta 1$) has been demonstrated to be important in the metastatic process of rhabdomyosarcoma cells [4], c) the vitronectin receptor α-chain (integrin αv subunit) seems to be involved in human melanoma tumorigenicity [8], and d) E-cadherin, a cell-cell adhesion molecule, can act as an invasion suppressor molecule in certain epithelial tumor cells [3,44]. Finally, intercellular adhesion molecule ICAM-1 [16,26], VLA-2 [2,19], and the vitronectin receptor β-chain [1] (integrin β3 subunit) have been shown to be preferentially expressed in vertical growth phase primary melanoma lesions and melanoma metastases suggesting a role in melanocytic tumor progression.

As only one study has been done with a large panel of monoclonal antibodies (mAbs) to look for integrin expression in melanocytic tumors [1], the purpose of this study was to investigate in a large number of lesions if there is a correlation between the stage of melanocytic tumor progression and the pattern of integrin expression.

MATERIAL AND METHODS

Lesions

Lesions were obtained from patients at the University Hospital, Nijmegen, The Netherlands and at the Department of Dermatology of the Wilhelms University, Münster, Germany. Based on histopathologic examination of paraffin sections, lesions were divided into five classes: common nevus nevocellularis (NN) (n=22; 3 compound, 10 junctional and 9 intradermal), dysplastic nevus [7,40] (atypical nevus; DN) (n=16), early primary melanoma (i.e. tumor thickness $\leq$ 1.5 mm; ePM) (n=24), advanced primary melanoma

(i.e. tumor thickness > 1.5 mm; aPM) (n=28) and melanoma metastasis (MM) (n=25). Representative samples were snap frozen in liquid nitrogen and stored at -80°C until sectioning.

Monoclonal antibodies

The following anti integrin mAbs were used: TS2/7 anti-α1 [12] (T-cell Sciences, Cambridge MA, USA); Gi-14 anti-α2 [34]; A1.43 anti-α2 [2,19]; J143 anti-α3 [9]; P1B5 anti-α3 [46] (Telios Pharmaceuticals Inc., San Diego CA, USA); HP2/1 anti-α4 [33] (Immunotech S.A., Marseille, France); P4G9 anti-α4 [48] (Telios Pharmaceuticals Inc., San Diego CA, USA); NKI-Sam1 anti-α5 [42]; P1D6 anti-α5 [47] (Oncogene Science Inc., Uniondale NY, USA); MT78 anti-α6 [20]; GoH3 anti-α6 [39]; NKI-M7 anti-αv [6]; SZ.22 anti-αIIb [31]; Aj2 anti-β1 [17]; 4B4 anti-β1 [24] (Coulter Immunology, Hialeah FL, USA); C17 anti-β3 [41]; and 3E1 anti-β4 [13] (Telios Pharmaceuticals Inc., San Diego CA, USA).

Immunohistochemical staining

Four μm cryostat sections were fixed in acetone for 10 minutes and incubated at room temperature with mAbs for 1 h. After washing with phosphate buffered saline (PBS) bound mAbs were visualized using the peroxidase based Vectastain elite ABC kit (Vector Laboratories, Burlingame CA, USA) and amino-ethyl carbazole (AEC). After counterstaining with Meyers hematoxylin sections were mounted with Kaisers glycerin/gelatin.

Score

The intensity of staining of the melanocytic cells was scored semiquantitatively as: negative, positive or strongly positive. The percentage of stained melanocytic cells was estimated as: 0, 1-5, 6-25, 26-50, 51-75, 76-100% (Fig 1). Slides were read independently by two observers. Discrepancies exceeding one percentage class were found in less than 10% of the cases. These cases were reevaluated jointly until consensus could be reached.

Logistic regression was used to detect antigen expression correlated with tumor progression.

RESULTS

The staining patterns for the various integrin subunits on different stages of melanocytic tumor progression are described below. Similar results were obtained for compound-, junctional- and intradermal nevi and these groups are therefore not mentioned separately.

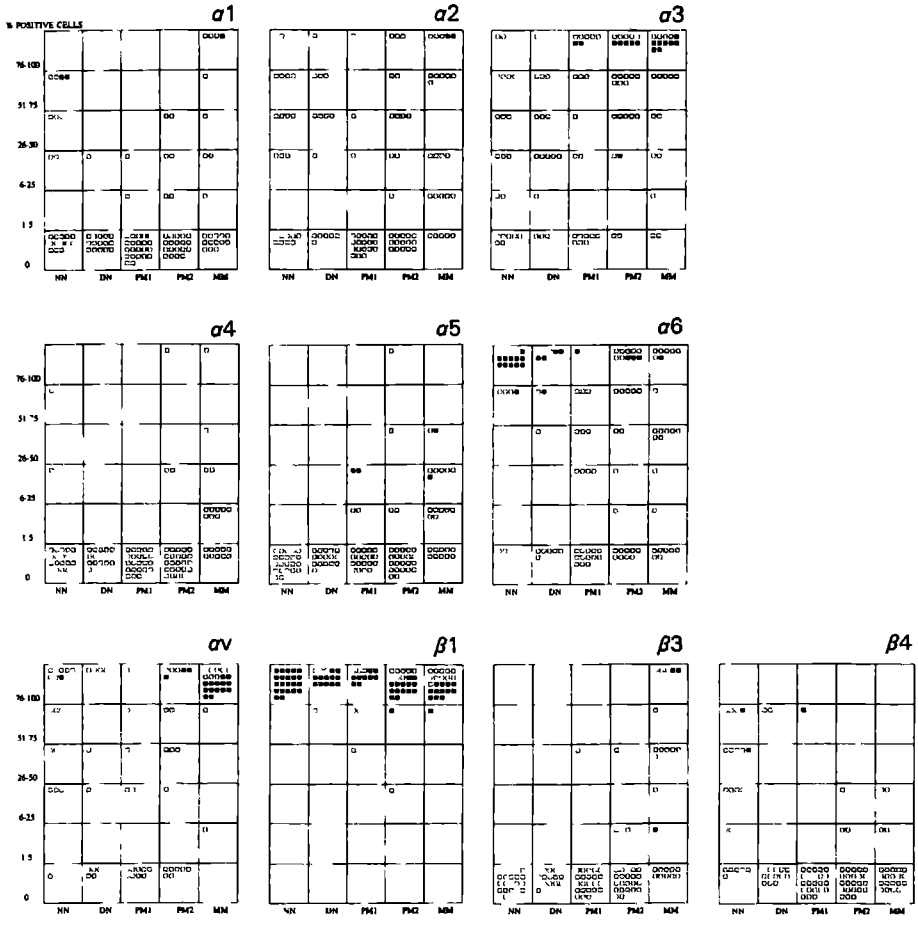


Figure 1. *Percentage of positive cells in each lesion stained with anti-integrin mAbs. NN=common nevus nevocellularis, DN=dysplastic nevus, PM1=early primary melanoma (ePM), PM2=advanced primary melanoma (aPM), MM=melanoma metastasis. Staining intensity was scored as positive (open square) or strongly positive (filled square).*

α1. Most lesions did not show detectable α1 expression. However, 25% of the lesions, both benign and malignant, stained with TS2/7 mAbs (Figs 1,2a). 9 out of 22 NN

(41%) were positive, whereas only 1 out of 16 DN was found to contain positive cells. 8% (2/24) of ePM and 24% (6/25) of aPM was found to be positive whereas 41% (9/22) of MM contained positive cells.

α2. All lesions were incubated with A1.43 and Gi14 anti-α2 mAbs; similar results were found with both mAbs. There was a marked difference between the number of positive ePM (14%; 3/21) and the number of positive aPM (44%; 12/27) and MM (80%; 20/25) (Fig 1). However, the number of positive NN (59%; 13/22) and DN (60%; 9/15) was in the same range as the number of positive aPM and MM. A membranous pattern of staining was observed in MM lesions whereas in NN, DN and PM this was not obvious (Fig 2b).

α3. Staining with J143 and P1B5 anti-α3 mAbs gave similar results. NN, DN, and ePM stained in variable percentages but staining was weak and 33% (7/21) of NN, 19% (3/16) of DN, and 38% (8/21) of ePM were negative (Fig 1). In the case of aPM and MM however, only few lesions were negative: 7% (2/27) and 8% (2/24) respectively. Furthermore a number of aPM and MM were strongly positive: 22% (6/27) and 33% (8/24) respectively. In contrast with nevi and PM, in MM usually a clear membranous staining pattern was observed (Fig 2c).

α4. Approximately 10% of NN (2/21) and aPM (3/27) showed α4 expression while no expression was detected in DN and ePM (Fig 1). In MM, 55% (12/22) of the lesions were found to stain positive with HP2/1 mAbs (not shown). Expression of α4 appears to increase in MM ($p=0.002$).

α5. Staining with P1D6 and NKI-SAM1 anti-α5 mAbs gave similar results but usually P1D6 stained less strongly than NKI-SAM1. In NN and DN, α5 expression could not be detected (Figs 1,2d). Approximately 15% of ePM (4/23) and aPM (4/26) stained whereas 60% (15/25) of MM were α5 positive. No obvious membranous pattern of staining was observed (Figs 2e,f). These results show that expression of α5 emerges during melanocytic tumor progression ($p=0.0001$).

α6. Staining with MT78 and GoH3 anti-α6 mAbs gave similar results. Almost all NN (19/21) were positive (Fig 1). Basal keratinocytes stained strongly at their basal surface whereas nevus cells showed no obvious membranous staining pattern (Fig 2g). Often, strong staining of keratinocytes was interrupted at the site of nevus cell nests. Even though not significant ($p>0.05$), more DN, ePM, aPM, and MM were negative; 38% (6/16), 54% (13/24), 32% (9/28) and 29% (7/24) respectively. Usually, staining in PM and MM was not strong (Fig 2h) whereas 57% of NN and 31% of DN was strongly positive.

αv. 72% of NN (16/22), 56% of DN (9/16), 40% of ePM (6/15), and 63% of aPM (12/19) stained with NKI-M7 anti-αv mAbs (Fig 1). All MM were positive and in all but two MM more than 75% of the cells stained. In addition, 58% (14/24) of MM stained strongly with NKI-M7. A partly cytoplasmic and membranous staining pattern was observed (Fig 2i). The αv subunit appears to be quantitatively upregulated during

melanocytic tumor progression (p=0.04).

αIIb. No αIIb expression was detected in any of the lesions stained with SZ.22 mAbs; internal control cells (platelets) were positive (not shown).

β1. In all lesions except 2 PM more than 50% of cells stained with AJ2 and 4B4 anti-β1 mAbs (Fig 1). In general the lesions showed strong expression (not shown).

β3. None of the NN and DN were stained positive with C17 anti-β3 mAbs (Fig 1,2j). Out of 24 ePM only one showed expression of the β3 subunit in 26-50% of the cells. 15% (4/26) of aPM and 58% (14/24) of MM stained positive with C17, three of which were strongly positive (Fig 2k). These results show that β3 emerges during melanoma progression (p=0.0001).

β4. 71% of NN (15/21) showed expression of the β4 subunit as detected with 3E1 mAbs whereas less then 20% of the lesions from each of the other categories was found to be positive (Fig 1,2l,m). For keratinocytes, staining was similar to that observed with anti-α6 mAbs (not shown). It appears that β4 expression decreases with melanocytic tumor progression (p=0.0003).

DISCUSSION

From this study and two others [1,27] in which integrin expression in fresh human melanocytic lesions was investigated, it is clear that during melanocytic tumor progression a number of changes in integrin expression occur. Albelda et al. [1] detected emerged expression of the β3 subunit in vertical growth phase PM and in MM, and Natali et al. [27] who studied only α6β1/β4 integrins found decreased expression of α6β1 to be a striking phenomenon in melanocytic tumor progression. In the present study, on a large number of cases and with a broad panel of mAbs we find both decreased and increased expression of integrins and also emergence of subunits which can not be detected in benign lesions, to occur during melanocytic tumor progression.

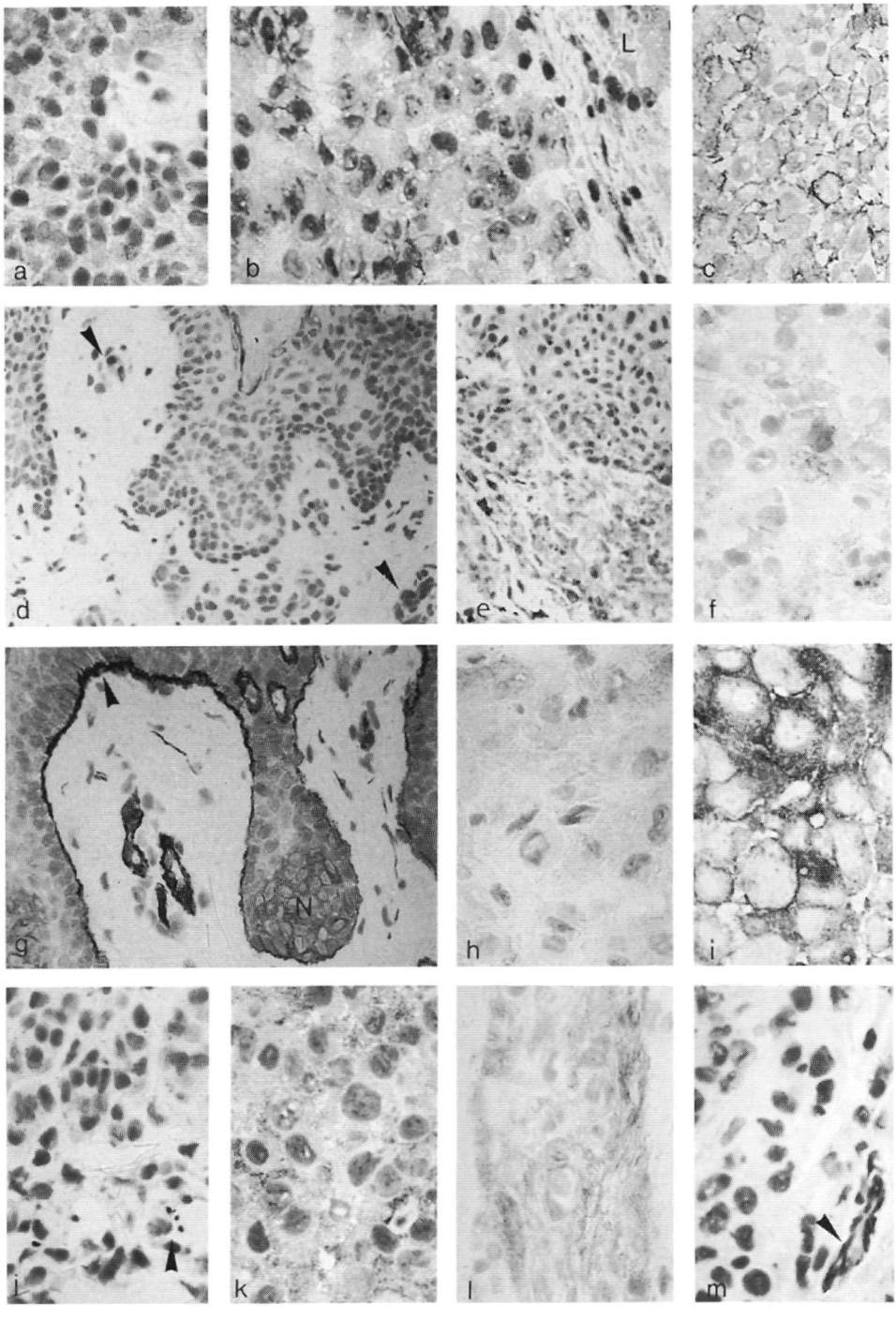
Our finding that almost all NN express α6 whereas expression is absent in 30 to 50% of PM and MM, is in line with findings from Natali et al. [27] who reported downregulation of α6 expression associated with invasiveness. However, the decrease of α6 expression during melanocytic tumor progression is not significant in our material and Albelda et al. [1] did not detect any correlation. These differences can not be explained by differences between the mAbs used as both Albelda et al. and we ued GoH3 anti-α6 and the mAb used by Natali et al. recognizes the same epitope on the α6 molecule [18]. It may be partly explained by differences in classification of PM, i.e. thickness versus growth phase, and possibly by different sensitivities of the methods used. The fact that β4 is present in NN and like α6, an increasing number of lesions is β4 negative with melanocytic tumor progression, may suggest that nevus cells express α6β4. The ligand for α6β4 has recently been demonstrated to be laminin [22] and α6β4 seems to play an

important role in maintaining the epidermal structure as it is involved in keratinocyte adhesion to the basal membrane [38]. In keratinocytes β4 is localized in hemidesmosomes [38] and we find strong β4 expression only on the basal side of these cells. The fact that the strong β4 signal usually found in keratinocytes, is not detected where nevus cells appear to be in close contact with the basal lamina may indicate that even though β4 is expressed in nevus cells it does not play a role as a receptor for the basal lamina similar to its role in keratinocytes.

Our finding that β3 emerges during melanocytic tumor progression is in line with findings reported by Albelda et al. [1]. The fact that we can not detect β3 expression in all of the MM may be explained by differences in the mAbs used or by the sensitivity of the methods. It may also be that β3 is not necessarily associated with melanocytic tumor progression though there are indications for a role for β3 in this process. Thus β3 has been demonstrated to play a role as a signal transducing molecule which is important for melanoma metastasis [35]. The fact that we detect αv expression in all lesions tested indicates that αv combines with other subunits than β3 in the benign lesions where β3 is absent. In fact, alternative β subunits have been shown to combine with αv [5,21,45]. Our finding that αv expression is upregulated in MM may indicate a role for αv in the metastatic process. There is evidence that αv is important in melanoma tumorigenesis [8] and it has also been implicated in the metastatic process of melanoma cells [10].

Our finding that α5 expression emerges in PM and is strongly increased in MM confirms and extends previous reports where α5 expression in melanoma was investigated: a) Mortarini et al. [25] did not detect any α5 expression on cells cultured from PM whereas cells cultured from two out of three MM expressed α5, and b) Albelda et al. [1] detected α5 expression only in vertical growth phase PM and in MM. These and our findings strongly suggest that expression of the classical fibronectin receptor VLA-5 [30], is associated with melanocytic tumor progression and that its role in melanoma cells is different from CHO cells where VLA-5 has been demonstrated to suppress rather than enhance the transformed phenotype [11].

Figure 2. *Immunohistochemical staining with anti-integrin mAbs on human melanocytic lesions.* ***A:*** *ePM stained with TS2/7 α1 mAbs.* ***B:*** *MM stained with A1.43 α2 mAbs. Note that no α2 is detected in the liver parenchyma (L).* ***C:*** *MM stained with J143 α3 mAbs.* ***D:*** *NN stained with NKI-Sam1 α5 mAbs. Some small vessels are positively stained (arrows).* ***E:*** *ePM stained with NKI-Sam1 α5 mAbs. Both regions of positive and negative melanoma cells were found.* ***F:*** *MM stained with NKI-Sam1 α5 mAbs.* ***G:*** *NN stained with GoH3 α6 mAbs. Note the strong basal staining of basal keratinocytes (arrow) and the cytoplasmic staining of nevus cells (N).* ***H:*** *MM stained with GoH3 α6 mAbs.* ***I:*** *MM stained with NKI-M7 αv mAbs.* ***J:*** *DN stained with C17 β3 mAbs. Some blood cells are positively stained (arrow).* ***K:*** *MM stained with C17 β3 mAbs.* ***L:*** *DN stained with 3E1 β4 mAbs.* ***M:*** *MM stained with 3E1 β4 mAbs. Blood vessel walls are positively stained (arrow).* *(see opposite page).*


a
b
L
c
d
e
f
g
N
h
i
j
k
l
m

To date, little is known about the expression of integrins in normal melanocytes in situ. One immunoelectronmicroscopic study on epithelial cell suspensions [49] demonstrates expression of $\alpha 3$ and $\alpha 6$ but not $\alpha 1$, $\alpha 2$, or $\alpha 4$ in normal melanocytes. We and others [1] find expression of $\alpha 1$ and $\alpha 2$ in NN which may indicate that formation of a nevus implies changes in the adhesive phenotype of melanocytes. The fact that we find increased $\alpha 4$ expression in MM but also in a small number of NN is in line with results reported by Albelda et al. [1]. In an earlier study, $\alpha 2$ expression has been described to correlate with melanocytic tumor progression [2]. In the present study we find strongly increased $\alpha 2$ expression in aPM and MM compared with ePM but a large number of nevi shows $\alpha 2$ expression as well. As in both studies the same mAbs (A1.43) are used, these differences may be due to different sensitivity of the methods used or the fact that in the earlier study weak staining was scored negative.

Finally, it is known that integrins can be present in an active or inactive state and that activation is often required for high affinity interaction with their ligands [28,37,42,43]. Therefore, the fact that changes in the expression of a number of integrins are observed during melanocytic tumor progression does not necessarily indicate that during this process cells loose or gain the functional properties known to be mediated by these integrins.

In conclusion, we find a number of alterations in integrin expression during melanocytic tumor progression: decrease of $\alpha 6$ and $\beta 4$, increase of $\alpha 4\beta 1$, and the most striking being emergence of the $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor. In this respect it is interesting that signaling through both $\alpha 5\beta 1$ [36] and $\alpha v\beta 3$ [35] can modulate the 72 kDa type IV collagenase during human melanoma cell invasion. Besides $\beta 3$ which has been described before, $\alpha 5$ may be useful as a marker for malignant melanocytic lesions.

ACKNOWLEDGEMENTS

We thank Drs. Carl Figdor, Eberhard Klein, S. Santoso, and Arnoud Sonnenberg for generously providing the antibodies and Mrs. José Aldeweireldt for expert technical assistance. This work was financally supported by the Dutch Cancer Society (Grant NUKC 91-09) and the European Community Concerted Action on melanoma progression.

REFERENCES

1. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: Association of the $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

2. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36, 29-35, 1985

3. Bussemakers MJG, Van Moorselaar RJA, Giroldi LA, Ichikawa T, Isaacs JT, Takeichi M, Debruyne FMJ, Schalken JA. Decreased expression of E-cadherin in the progression of rat prostatic cancer. Cancer Res 52, 2916-2922, 1992.

4. Chan BMC, Matsuura N, Takada Y, Zetter BR, Hemler ME. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. Science 251, 1600-1602, 1991.

5. Cheresh D, Smith J, Cooper H, Quaranta V. A novel vitronectin receptor integrin ($\alpha v\beta x$) is responsible for distinct adhesive properties of carcinoma cells. Cell 57, 59-69, 1989.

6. De Vries JE, Keizer GD, te Velde AA, Voordouw A, Ruiter DJ, Rümke P, Spits H, Figdor CG. Characterization of melanoma-associated surface antigens involved in the adhesion and motility of human melanoma cells. Int J Cancer 38, 465-473, 1986.

7. De Wit PEJ, Van 't Hof-Grootenboer B, Ruiter DJ, Bondi R, Bröcker E-B, Cesarini JP, Hastrup N, Hou-Jensen K, MacKie RM, Scheffer E, Suter L, Urso C. Validity of the histopathological criteria used for diagnosing dysplastic naevi. Eur J Cancer, 29A, 831-839, 1993.

8. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.

9. Fradet Y, Cordon-Cardo C, Thomson T, Daly ME, Whitmore WFJr, Lloyd KO, Melamed MR, Old LJ Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies Proc Natl Acad Sci USA 81, 224-228, 1984.

10. Gehlsen KR, Davis GE, Sriramarao P. Integrin expression in human melanoma cells with differing invasive and metastatic properties. Clin Exp Metastasis 10, 111-120, 1992.

11. Giancotti FG, Ruoslahti E. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells Cell 60, 849-859, 1990.

12. Hemler ME, Sanchez-Madrid F, Flotte TJ, Krensky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL. Glycoproteins of 210,000 and 130,000 m.w. on activated T-cells· Cell distribution and antigenic relation to components on resting cells and T cell lines. J Immunol 132, 3011-3018, 1984.

13. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E Basement membrane diversity detected by monoclonal antibodies. Differentiation 26, 49-54, 1984.

14. Hynes RO. Integrins: A family of cell surface receptors. Cell 48, 549-554, 1987.

15. Hynes RO. Integrins· Versatility, modulation and signalling in cell adhesion. Cell 69, 11-25, 1992.

16. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmüller G. De novo expression of intercellular adhesion molecule-1 in melanoma correlates with increased risk of metastasis Proc Natl Acad Sci USA 86, 641-644, 1989.

17. Kantor RRS, Mattes MJ, Lloyd KO Biochemical analysis of two cell surface glycoprotein complexes, very common antigen 1 and very common antigen 2. Relationship to very late activation T cell antigens J Biol Chem 262, 15158-15165, 1987

18. Kennel SJ, Foote LJ, Falcioni R, Sonnenberg A, Stringer CD, Crouse C, Hemler ME Analysis of the tumor associated antigen TSP-180. Identity with $\alpha 6\beta 4$ in the integrin superfamily J Biol Chem 264, 15515-15521, 1989.

19. Klein CE, Steinmeyer T, Kaufmann R, Weber L, Bröcker E-B. Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96, 281-284, 1991.

20. Klein CE, Steinmeyer T, Mattes J, Kaufmann R, Weber L. Integrins of normal human epidermis differential expression, synthesis and molecular structure. Br J Dermatol 123, 171-178, 1990.

21. Krissansen GW, Elliot MJ, Lucas CM, Stomski FC, Berndt MC, Cheresh DA, Lopez AF, Burns GF Identification of a novel integrin β subunit expressed on cultured monocytes (macrophages) J Biol Chem

265, 823-830, 1990.
22. Lee EC, Lotz MM, Steele GDJr, Mercurio AM The integrin α6β4 is a laminin receptor J Cell Biol 117, 671-678, 1992.
23. Liotta L. Tumor invasion and metastasis - role for the extracellular matrix. Rhoads memorial award lecture. Cancer Res 46, 1-7, 1987.
24. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.
25. Mortarini R, Anichini A, Parmiani G. Heterogeneity for integrin expression and cytokine-mediated VLA modulation can influence the adhesion of melanoma cells to extracellular matrix proteins Int J Cancer 47, 551-559, 1991.
26. Natali P, Nicotra M, Cavaliere R, Bigotti A, Romano G, Temponi M, Ferrone S. Differential expression of intercellular adhesion molecule 1 in primary and metastatic melanoma lesions. Cancer Res 50, 1271-1278, 1990.
27. Natali PG, Nicotra MR, Cavaliere R, Giannarelli D, Bigotti A. Tumor progression in malignant melanoma is associated with changes in α6β1 laminin receptor. Int J Cancer 49, 168-172, 1991.
28. O'Toole TE, Loftus JC, Du X, Glass A, Ruggieri ZM, Shattil SJ, Plow EF, Ginsberg MH. Affinity modulation of the αIIbβ3 integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor Cell Reg 1, 883-893, 1990.
29. Poste G, Fidler IJ. The pathogenesis of cancer metastasis Nature 283, 139-146, 1980
30. Pytela R, Pierschbacher MD, Ruoslahti E. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. Cell 40, 191-198, 1985
31. Ruan CG, Xi XD, Du XP, Wan HY, Wu X, Li PX, Gu JM. Studies on monoclonal antibodies against human platelets - a monoclonal antibody against human platelet glycoprotein I SZ-2. Sci Sin 30, 404-412, 1987.
32. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion. RGD and integrins Science 238, 491-497, 1987.
33. Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C VLA-3· A novel polypeptide association within the VLA molecular complex Cell distribution and biochemical characterization. Eur J Immunol 16, 1343-1349, 1986.
34. Santoso S, Kiefel V, Müller-Eckhardt. Immunochemical characterization of the new platelet alloantigen system. Br J Haematol 72, 191-198, 1989.
35. Seftor EB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the αvβ3 integrin in tumor cell invasion Proc Natl Acad Sci USA 89, 1557-1561, 1992.
36. Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC The 72 kDa type IV collagenase is modulated via differential expression of αvβ3 and α5β1 integrins during human melanoma cell invasion Cancer Res 53, 3411-3415, 1993.
37. Shimizu Y, Van Seventer GA, Horgan KJ, Shaw S. Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature 345, 250-253, 1990.
38. Sonnenberg A, Calafat J, Janssen H, Daams H, Van der Raaij-Helmer LMH, Falcioni R, Kennel SJ, Aplin JD, Baker J, Loizidou M, Garrod D Integrin α6β4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion J Cell Biol 113, 907-917, 1991.
39. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. J Biol Chem 264, 13745-13750, 1987.
40. Steijlen PM, Bergman W, Hermans J, Scheffer E, Van Vloten WA, Ruiter DJ The efficacy of histopathological criteria required for diagnosing dysplastic naevi Histopathology 12, 289-300, 1988.
41. Tetteroo PAT, Lansdorp PM, Leeksma OC, Von dem Borne AEG. Monoclonal antibodies against

human platelet glycoprotein IIIa. Br J Haematol 55, 509-51, 1983.

42. Van de Wiel-van Kemenade E, Van Kooyk Y, De Boer AJ, Huijbens RJF, Weder P, Van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

43. Van Kooyk YP, Van de Wiel-van Kemenade E, Weber P, Kuijpers TW, Figdor CG. Enhancement of LFA-1 mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature 342, 811-813, 1989.

44. Vleminckx K, Vakaet L, Mareel M, Fiers W, Van Roy F. Genetic manipulation of E-cadherin expression by epithelial cells reveals an invasion suppressor role. Cell 66, 107-119, 1991.

45. Vogel B, Tarone G, Giancotti FG, Gailit J, Ruoslahti EA. A novel fibronectin receptor with an unexpected subunit composition ($\alpha v\beta 1$). J Biol Chem 265, 5934-5937, 1990.

46. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. J Cell Biol 105, 1873-1884, 1987.

47. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. The function of multiple extracellular matrix receptors in mediating adhesion to extracellular matrix: Preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion of fibronectin and react with platelet glycoproteins Ic/IIa. J Cell Biol 107, 1881-1891, 1988.

48. Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG. Identification and characterization of the lymphocyte adhesion receptor for an alternative cell attachment domain in plasma fibronectin. J Cell Biol 109, 1321-1330, 1989.

49. Zambruno G, Manca V, Santantonio ML, Soligo D, Gianetti A. VLA protein expression on epidermal cells (keratinocytes, Langerhans cells, melanocytes): A light microscopic immunohistochemical study. Brit J Dermatol 124, 135-145, 1991.

Chapter 5

Integrin expression in uveal melanoma differs from cutaneous melanoma

Invest Ophthalmol Vis Sci **34**, 3635-3640, 1993

Integrin expression in uveal melanoma differs from cutaneous melanoma

Paul JM ten Berge[1], Erik HJ Danen[1], Goos NP van Muijen[1], Martine J Jager[2], and Dirk J Ruiter[1]

[1]The Department of Pathology, University Hospital, Nijmegen, and [2]The Department of Ophthalmology, University Hospital, Leiden, The Netherlands

During the process of metastasis changes in cell-cell and cell-matrix contacts occur and expression of integrins, a superfamily of adhesion molecules, may therefore be important. Expression of integrins has been extensively studied in cutaneous melanoma. Since it is known that uveal melanoma has a metastatic behavior different from cutaneous melanoma, we investigated integrin expression in uveal melanoma. We employed monoclonal antibodies recognizing integrin subunits α1-6, αv, β1, β4, and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ on frozen sections of 32 human primary uveal melanomas and 4 metastases, followed by an ABC-immunoperoxidase technique. As in cutaneous melanoma α4 expression was rare whereas most lesions expressed α3 and α6. In contrast to cutaneous melanoma where α2 is well expressed in most lesions and α5 is expressed only in a low percentage of primary lesions, in uveal melanoma α2 expression was rare whereas α5 expression was found in all lesions. A major difference was observed with regard to the $\alpha v\beta 3$ vitronectin receptor. In contrast to cutaneous melanoma where $\alpha v\beta 3$ is expressed in advanced primary lesions, $\alpha v\beta 3$ was not detected in any of the primary uveal melanomas, irrespective of the thickness or cell type of the tumor. All lesions strongly expressed $\alpha v\beta 5$. Thus, integrin expression in uveal melanoma cannot be correlated with either cell type or invasiveness, and in contrast with cutaneous melanoma, determination of the integrin expression profile seems not to be suitable to subdivide uveal melanomas into low and highly malignant lesions.

INTRODUCTION

In contrast to cutaneous melanoma, uveal melanoma metastasizes primarily to the liver. This difference in biological behavior cannot be explained on the basis of any known anatomic or physiologic factors [6]. Other sites where uveal melanomas may metastasize to are the brains, lungs, bone marrow, lymph nodes, pericardium, skin, and organs of the gastro-intestinal tract. As the eye lacks lymphatics, unlike cutaneous melanoma, uveal melanoma does not exhibit direct lymphatic spread. Besides direct hematogenous spread, uveal melanoma cells can invade the sclera and reach the orbital tissues, usually at sites where blood vessels and possibly lymph vessels pass through.

In order for malignant cells to detach from their primary location, to attach to extracellular matrix (ECM) components of the surrounding stroma, to enter a blood vessel, and to metastasize, their adhesive properties must change repeatedly [18]. Receptors mediating cell adhesion may therefore be of great importance to metastasis [15,19]. Integrins, a family of cell-surface receptors, participate in cell adhesion and migration [11]. These heterodimeric glycoproteins consist of an α-chain noncovalently linked to a β-chain, rendering ligand specificity. Integrins are divided into families on the basis of a common β-chain. So far three subfamilies (β1, β2, β3) have been extensively studied and 5 additional β-subunits have been described. In cutaneous melanocytic lesions the pattern of integrin expression has been reported to correlate with tumor progression [1,2,4,12,17].

As uveal melanoma differs biologically and clinically from cutaneous melanoma, uveal melanoma may express a different pattern of integrins. In this study we therefore investigated the expression pattern of a number of integrins in human uveal melanoma, using a panel of monoclonal antibodies (mAbs) for immunohistochemical staining of α1-6, αv, β1, β4, αvβ3 and αvβ5 in frozen specimens.

MATERIALS AND METHODS

Lesions

Specimens of 32 primary uveal melanomas and 4 uveal melanoma metastases were obtained from patients at the Nijmegen University Hospital, the Leiden University Hospital, the Rotterdam University Hospital, the Academical Medical Center, Amsterdam, and the Foundation of Deventer Hospitals, The Netherlands. Methods for securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki. Diagnosis of primary uveal melanomas was microscopically assessed on paraffin sections, discerning spindle (n=11), mixed (n=14), and epithelioid (n=7) cell types. Invasion of the sclera was graded as not (n=3), superficially (less than 25% of scleral thickness) (n=11), half (ca. 50% of scleral

thickness) (n=5), deep (ca. 75% of scleral thickness) (n=6), and episclerally (n=4) invaded. Bruch's membrane was regarded intact (n=9) or ruptured (n=20). From 3 primary uveal melanomas invasion of sclera or Bruch's membrane could not be determined. Representative parts of all specimens were embedded in Tissue Tek OCT compound (Ames Company, Division of Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen. In a cryostat 4μm sections were cut serially at -25°C, mounted on uncoated slides and stored at -80°C until use.

Table 1. Monoclonal antibodies.

Integrin	mAb	Source and reference
α1	TS2/7	T-cell Science, Cambridge, MA [9]
α2	Gi14	Dr Santoso, Giessen, Germany [21]
α2	A1.43	Dr Bröcker, Würzburg, Germany [2,12]
α3	P1B5	Telios, San Diego, CA [26]
α4	HP2/1	Immunotech, Marseille, France [20]
α5	NKI-Sam1	Dr Figdor, Amsterdam, The Netherlands [25]
α5	P1D6	Oncogene Science, Uniondale, NY [27]
α6	GoH3	Dr Sonnenberg, Amsterdam, The Netherlands [24]
α6	MT78	Dr Klein, Würzburg, Germany [13]
αv	NKI-M7	Dr Figdor, Amsterdam, The Netherlands [5]
β1	4B4	Coulter, Hialeah, FL [16]
αvβ3	LM609	Dr Cheresh, La Jolla, CA [3]
αvβ5	P1F6	Gibco, Gaithersburg, MD [28]
β4	3E1	Telios, San Diego, CA [10]

Immunohistochemistry

Integrin expression was detected by an indirect immunoperoxidase method using a panel of mAbs listed in Table 1. In brief, sections were air dried, fixed in acetone for 10 min, and incubated with the mAbs at room temperature for 60 min. MAbs were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and optimal working dilutions had been determined on positive controls previously. After PBS rinsing, detection of the primary antibodies was achieved using the peroxidase-based Vectastain elite ABC system (Vector Laboratories, Burlingame, CA). This consisted of a biotinylated rabbit anti-rat Ig for mAb GoH3 and a biotinylated horse anti-mouse Ig for all other mAbs, followed by an avidin-biotin-peroxidase complex (ABC). As a substrate 3-amino-

9-ethyl-carbazole (AEC) in acetate buffer pH 4.85 containing hydrogen peroxide was used. Finally, sections were counterstained with methyl green or hematoxylin and mounted with Kaiser's glycerol/gelatin (Merck, Darmstadt, Germany). Negative controls consisted of incubations replacing the primary antibodies by PBS/BSA.

Score

The intensity of staining of the melanocytic cells was scored semiquantitatively as: negative, positive or strongly positive. The percentage of stained melanocytic cells was estimated as: 0, 1-5, 6-25, 26-50, 51-75, 76-100%. Slides were read independently by two observers. Discrepancies exceeding one percentage class were found in less than 10% of the cases. These cases were reevaluated jointly until consensus was reached.

RESULTS

Staining results for the seven α subunits and four β-subunits showed a consistent expression pattern among the 32 primary uveal melanomas (Fig 1).

Uniformly positive staining was observed in all lesions when incubated with mAbs against the common β subunit of the β1 integrins. A low proportion of lesions stained with mAbs against the α1 (5/32, 16%), α2 (3/32, 9%) and α4 (8/32, 25%) subunits. When expression of these integrin subunits was detected it usually was of moderate intensity and a low percentage of tumor cells stained. A1.43 and Gi14 anti-α2 mAbs showed identical staining patterns (not shown).

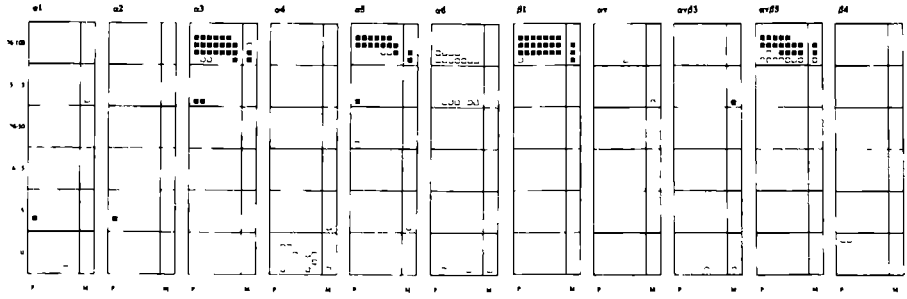


Figure 1. Percentage of positive cells in lesions stained with anti-integrin mAbs. P, primary uveal melanoma; M, uveal melanoma metastasis. Open and closed squares indicate moderately and strongly positive lesions respectively.

Expression of the α6 subunit was found in a majority of lesions (24/32, 75%) though a large variability in the number of positive tumor cells was observed; i.e. in 8/32 lesions no positive tumor cells could be detected (Fig 2c), whereas in 11/32 lesions approximately all melanoma cells were stained though staining was usually cytoplasmic and of moderate intensity (Fig 2d). A marked difference in reactivity was seen between GoH3 and MT78 anti-α6 mAbs, as the latter detected expression in only two lesions (not shown).

All lesions stained with mAbs directed against α3 (Fig 2a), α5 (Fig 2b), and αv (not shown). Usually a high percentage of tumor cells within a given lesion stained strongly with these mAbs though staining with NKI-M7 anti-αv mAbs was of moderate intensity. Expression of α5 could always be detected with NKI-SAM1 mAbs, but with P1D6 mAbs only few lesions stained (not shown).

The β4 subunit could not be detected on melanoma cells in any of the lesions though staining of bloodvessels was observed with 3E1 anti-β4 mAbs (not shown).

No expression of αvβ3 was observed in any of the primary lesions though staining of bloodvessels was observed with LM609 anti αvβ3 mAbs (Fig 2e). In contrast all lesions strongly expressed αvβ5 (Fig 2f).

Similar to primary uveal melanoma, the metastatic lesions expressed the β1-subunit and none of them expressed the β4 subunit (Fig 1). Furthermore, the metastatic lesions expressed α3, α5, and αv and 3/4 expressed α6. In contrast to primary uveal melanoma, all metastatic lesions showed α2 and 3 out of 4 expressed α4 though a low percentage of cells within a given lesion stained and staining was of moderate intensity. Similar to the results in primary uveal melanoma, 76-100% of the cells in all three uveal melanoma metastasis lesions expressed αvβ5. In two lesions strong staining was observed and in one lesion staining was of moderate intensity (Fig 2h). Expression of αvβ3 which was not detected in any of the primary lesions, could be detected in most cells of one of the metastatic lesions (Fig 2g) and in some cells of another metastatic lesion.

Some sections from one of the metastatic lesions showed mainly necrotic tumor tissue and no more material was available. Therefore, staining with anti-α3, -α5, -β1, -αvβ3, -αvβ5, and -β4 mAbs could be investigated in only three metastatic lesions.

Within our series of primary tumors neither the cell type nor the invasion of sclera or Bruch's membrane correlated with an expression preference for any of the integrin-subunits.

DISCUSSION

Integrins in cutaneous melanoma have been the subject of a number of studies investigating the pathogenesis of metastasis [8]. Clinical and biological differences between cutaneous and uveal melanoma led us to the question whether different integrin

expression patterns could be found for these tumors. To the best of our knowledge this study is the first to investigate the expression pattern of a large number of integrins in human uveal melanoma. In our panel of primary uveal melanomas all cell types and stages are represented. As patients with metastases of uveal melanoma usually die at home, it is difficult to collect a set of metastases. Nevertheless we gathered 4 uveal melanoma metastases, but none located in the liver. Due to this low number, no significant conclusions can be drawn regarding integrin expression in uveal melanoma metastasis. The results of our survey have no diagnostic nor prognostic implications, though they may enhance understanding of biological processes with respect to uveal melanoma.

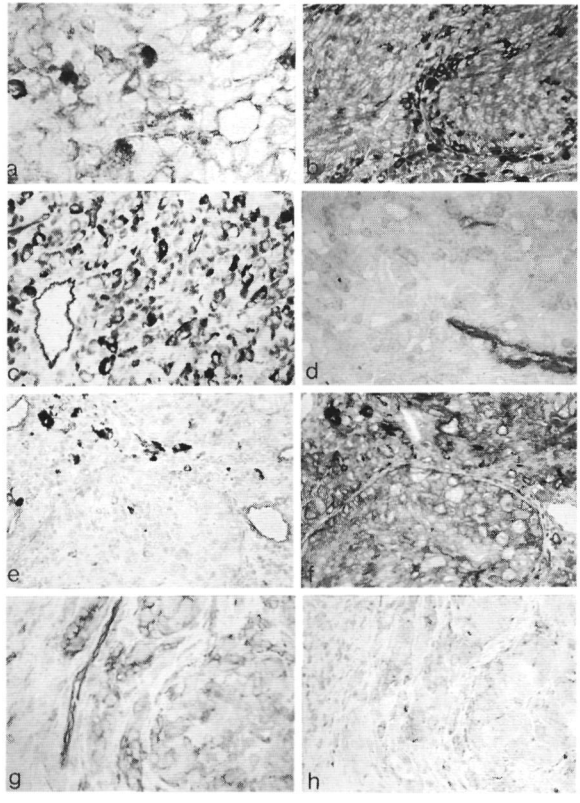


Figure 2. *Uveal melanomas stained with anti-integrin mAbs.* ***A:*** *Primary uveal melanoma stained with P1B5 anti-α3.* ***B:*** *Primary uveal melanoma stained with NKI-Sam1 anti-α5.* ***C,D:*** *Primary uveal melanoma lesions stained with GoH3 anti-α6. Note that C contains no positive tumor cells (vessel walls stain strongly) whereas in D all melanoma cells are positive.* ***E,F:*** *Primary uveal melanoma stained with (E) LM609 anti-αvβ3, or with (F) P1F6 anti-αvβ5. Identical regions in sequential sections are shown.* ***G,H:*** *Uveal melanoma metastasis stained with (G) LM609 anti-αvβ3, or with (H) P1F6 anti-αvβ5. Identical regions in sequential sections are shown.*

Comparison of integrin expression in this study and in studies on cutaneous melanoma indicates some differences. In uveal melanoma we find that all lesions express $\alpha 5$ but only a few are $\alpha 2$ positive whereas most cutaneous melanoma lesions express the $\alpha 2$-subunit and $\alpha 5$ can only be detected in about 20% of them [1]. Furthermore, even though no $\beta 4$ can be detected in uveal melanoma, a small number of cutaneous melanomas express this subunit [17]. The fact that only few lesions are $\alpha 4$ positive whereas $\alpha 3$, $\alpha 6$, and αv can be detected in the majority of lesions is similar to the findings in cutaneous melanoma [1,17]. The different staining profile using different mAbs against the same integrin subunit, as seen with mAbs against $\alpha 5$ and $\alpha 6$, is most probably due to differences in avidity of the mAbs or masking of specific epitopes. We have recently confirmed and extended the data on integrin expression in cutaneous melanoma in a large set of 115 melanocytic lesions [4]. The most striking difference between cutaneous and uveal melanoma concerns the $\alpha v\beta 3$ vitronectin receptor. In cutaneous melanoma $\alpha v\beta 3$ is expressed in metastases and in vertical growth phase primary lesions but not in nevi or in the relatively benign radial growth phase primary lesions [1]. In this study on uveal melanoma no $\alpha v\beta 3$ expression can be detected in any of the primary lesions, including those which may have metastatic potential such as those of the mixed and epithelioid cell types where Bruch's membrane is broken and sclera invaded. This indicates that the αv-subunit in uveal melanoma combines with an alternative subunit. We show that $\alpha v\beta 5$ is the vitronectin receptor expressed in primary uveal melanoma. In uveal melanoma metastasis both $\alpha v\beta 3$ and $\alpha v\beta 5$ may be expressed. It is known that αv can combine with $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, or $\beta 8$ [11]. Since no mAbs recognizing the $\alpha v\beta 1$, $\alpha v\beta 6$, or $\alpha v\beta 8$ complexes were available, we can not exclude the possibility that either of these integrins are present in uveal melanoma as well. Integrin $\alpha v\beta 5$ appears to be restricted to vitronectin in its ligand binding specificity whereas $\alpha v\beta 3$ interacts with multiple ligands [23]. Furthermore, $\alpha v\beta 3$ and $\alpha v\beta 5$ promote distinct cellular responses to vitronectin in vitro [14]. These findings indicate that the difference in expression of αv-integrins between cutaneous and uveal melanoma may lead to a different biological behavior. Since αv-integrins are thought to play a role in both proliferation [7] and invasion [22] of melanoma cells, the lack of $\alpha v\beta 3$ in primary uveal melanoma may have consequences for its growth and for its metastatic behavior. The fact that histological differences in cell type, and scleral and Bruch's membrane invasion cannot be correlated with a difference in integrin expression suggests that determination of the integrin expression profile is not suitable to subdivide uveal melanomas into low and highly malignant lesions. In contrast, this seems possible in cutaneous melanomas [1,4,17].

In conclusion we find integrin expression in human uveal melanoma not to be correlated with either cell type or invasiveness. Furthermore, we find that the $\alpha v\beta 3$ vitronectin receptor, which is associated with cutaneous melanoma progression, is not expressed in primary uveal melanoma. In uveal melanoma we demonstrate that the αv subunit combines with $\beta 5$.

ACKNOWLEDGEMENTS

We thank Drs. Eva-Bettina Bröcker, David Cheresh, Carl Figdor, Eberhard Klein, S Santoso, and Arnoud Sonnenberg for generously providing the antibodies. We kindly thank Drs Cornelia Mooy, Jan Weening, and Evert Weltevreden for providing some of the lesions. Furthermore we like to thank Mrs. José Aldeweireldt for expert technical assistance. This work was supported by the Dutch Cancer Society (grant NUKC 91-09).

REFERENCES

1. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck, CA. Integrin distribution in malignant melanoma:Association of the $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

2. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36, 29-35, 1985.

3. Cheresh DA, Harper JR. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDa α-subunit. J Biol Chem 262, 1434-1437, 1987.

4. Danen EHJ, ten Berge PJM, van Muijen GNP, Bröcker E-B, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin receptor and $\alpha v\beta 3$ vitronectin receptor in melanocytic tumor progression. Histopathol 24, 249-256, 1994.

5. De Vries JE, Keizer GD, te Velde AA, Figdor CG. Characterization of melanoma-associated surface antigens involved in the adhesion and motility of human melanoma cells. Int J Cancer 38, 465-473, 1986.

6. Einhorn LH, Burgess MA, Vallejos C. Advanced metastatic malignant melanoma. Prognostic correlations and response to treatment in 426 patients. Cancer Res 34, 1995-2004,1974.

7. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.

8. Hart IR, Birch M, Marshall JF. Cell adhesion receptor expression during melanoma progression and metastasis. Cancer Metastasis Rev 10, 115-128,1991.

9. Hemler ME, Sanchez-Madrid F, Flotte TJ, Krensky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL. Glycoproteins of 210,000 and 130,000 m.w. on activated T-cells·Cell distribution and antigenic relation to components on resting cells and T-cell lines. J Immunol 132, 3011-3018, 1984.

10. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Basement membrane diversity detected by monoclonal antibodies. Differentiation 26, 49-54, 1984.

11. Hynes RO. Integrins:Versatility, modulation and signalling in cell adhesion. Cell 69, 11-25, 1922.

12. Klein CE, Steinmeyer T, Kaufmann D, Weber L, Bröcker EB Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96, 281-284, 1991.

13. Klein CE, Steinmeyer T, Mattes J, Kaufmann R, Weber L. Integrins of normal human epidermis:Differential expression, synthesis and molecular structure. Br J Dermatol 123, 171-178, 1990

14. Leavesley DI, Ferguson GD, Wayner EA, Cheresh DA. Requirement of the integrin $\beta 3$ subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J Cell Biol 117, 1101-1107, 1992.

15. Liotta L. Tumor invasion and metastasis--role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res 46, 1-7, 1987.

16. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.

17. Natali PG, Nicotra MR, Cavaliere R, Giannarelli D, Bigotti A. Tumor progression in malignant

melanoma is associated with changes in $\alpha 6\beta 1$ laminin receptor. Int J Cancer 47:168-172, 1991.

18. Poste G, Fidler Y. The pathogenesis of cancer metastasis. Nature 283, 139-146, 1980.

19. Ruoslahti E, Giancotti FG. Integrins and tumor cell dissemination. Cancer Cells 1, 119-126, 1989.

20. Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-3: A novel polypeptide association within the VLA molecular complex:Cell distribution and biochemical characterization. Eur J Immunol 16, 1343-1349, 1986.

21. Santoso S, Kiefel V, Müller-Eckhardt. Immunochemical characterization of the new platelet alloantigen system. Br J Haematol 72, 191-198, 1989.

22. Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the $\alpha v\beta 3$ integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 89, 1557-1561, 1992.

23. Smith JW, Vestal DJ, Irwin SV, Burke TA, Cheresh DA. Purification and functional characterization of integrin $\alpha v\beta 5$: an adhesion receptor for vitronectin. J Biol Chem 265, 11008-11013, 1990.

24. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. J Biol Chem 264, 13745-13750, 1987.

25. Van de Wiel-van Kemenade E, Van Kooyk Y, De Boer AJ, Huijbers RJF, Weder P, Van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

26. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. J Cell Biol 105, 1873-1884, 1987.

27. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. The function of multiple extracellular matrix receptors in mediating adhesion to extracellular matrix: Preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion of fibronectin and react with platelet glycoproteins IcIIa. J Cell Biol 107, 1881-1891, 1988.

28. Wayner EA, Orlando RA, Cheresh DA. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J Cell Biol 113, 919-929, 1991.

Chapter 6

Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and non- and highly metastatic melanoma cells

Int J Cancer **54**, 315-321, 1993

Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and non- and highly metastatic melanoma cells

Erik HJ Danen[1], Goos NP van Muijen[1], Elly van de Wiel-van Kemenade[2], Kees FJ Jansen[1], Dirk J Ruiter[1], and Carl G Figdor[2]

[1]Department of Pathology, University Hospital, Nijmegen, and [2]Department of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

We compared integrin-mediated adhesion to extracellular matrix (ECM) components of cultured human melanocytes and six human melanoma cell lines with different metastatic capacities in nude mice. Cultured melanocytes, and most melanoma cell lines adhered strongly to fibronectin (Fn), whereas only highly metastatic cell lines adhered to laminin (Ln) and collagen type one (CoI) and type four (CoIV). Adhesion to Ln was blocked by mAbs to $\alpha 2$ and $\alpha 6$, and adhesion to Co by mAbs to $\alpha 2$. This observation was consistent with the finding that expression of Ln receptor $\alpha 6\beta 1$ and Ln/Co receptor $\alpha 2\beta 1$ was low on melanocytes and non - or poorly metastatic cell lines whereas these integrins were strongly expressed on highly metastatic cell lines. In addition, immunoprecipitation from [^{35}S]-methionine labeled cells demonstrated increased synthesis of $\alpha 6$, $\alpha 2$, and $\beta 1$ in highly metastatic cell lines, and immunohistochemistry showed expression of $\alpha 6\beta 1$ and $\alpha 2\beta 1$ only in xenograft lesions from highly metastatic cell lines. Furthermore, the observation that $\alpha 2\beta 1/\alpha 6\beta 1$-mediated adhesion of melanocytes and non - or poorly metastatic cell lines to Co/Ln could be induced with stimulatory anti-$\beta 1$ mAbs, demonstrates that these receptors, on these cells, are expressed in an inactive state. Our results suggest that $\alpha 2\beta 1$ and $\alpha 6\beta 1$ may play a role in human melanoma metastasis in nude mice and demonstrate that interactions of these integrins with their ligands can be regulated at the level of synthesis, surface expression, and activation state of the receptor.

INTRODUCTION

Crucial steps in the process of metastasis are the release and migration of cells from the primary tumor, penetration of the vessel wall, arrest in the microcirculation of distant organs and subsequent extravasation [29]. During this process the malignant cells interact several times with the ECM components of basal lamina and stroma including Ln, Co, and Fn through specific receptors on the cell membrane termed integrins.

Integrins consist of a family of transmembrane receptors. Structurally each integrin is a heterodimer consisting of an α subunit noncovalently bound to a β subunit. Based on the type of β subunit present in the dimer the integrin family can be subdivided into several groups [17]. Most extensively studied are the $\beta 1$, the $\beta 2$, and the $\beta 3$ integrins. Beta-1 integrins (very late antigens, VLA's) play an important role in mediating cell-ECM contacts, $\beta 2$ integrins are exclusively expressed on leukocytes and mediate cell-cell contacts, whereas $\beta 3$ integrins have a broader distribution and mediate cell-ECM interactions. In order to mediate their functions integrins have to be in an activated state. This has been shown for $\beta 1$ [31], $\beta 2$ [39], and $\beta 3$ integrins [13].

Integrin expression and adhesive behavior of cells can change after transformation. F2408 rat fibroblast and normal rat kidney (NRK) cells show reduced adhesion to Fn after viral transformation [28] and certain human osteosarcoma cells show a changed pattern of integrin expression and of adhesive properties after further chemical transformation [8]. Highly metastatic tumor cells have been shown to be more adherent to basement membrane than tumor cells with low metastatic capacity [26]. Transfection of the $\alpha 5$ gene has been demonstrated to suppress the transformed phenotype of Chinese hamster ovary (CHO) cells [11] whereas transfection of the αv gene in M21 human melanoma cells that lack $\alpha v\beta 3$ expression restored their tumorigenicity [9] and transfection of the $\alpha 2$ gene has demonstrated the importance of VLA-2 in the metastatic process of rat rhabdomyosarcoma cells [6]. Finally, expression of $\alpha 2\beta 1$ [4,21], the $\beta 3$ subunit [1], and intercellular adhesion molecule ICAM-1 [18], a ligand for integrin $\alpha l\beta 2$, has been shown to be related to melanocytic tumor progression.

The purpose of this study is to investigate whether a correlation exists between the metastatic potential of human melanoma cell lines and their capacity to adhere to ECM components, and to study the mechanism of adhesion.

MATERIAL AND METHODS

Cell lines and culture conditions

The melanoma cell lines used, included: IF6 [41], 530 [43], M14 [19], Mel57 [5], BLM [41], and MV3 [42]. All cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Flow laboratories, Irvine, UK) supplemented with 10% fetal calf

serum (FCS), gentamycin, glutamate and pyruvate. Isolation and propagation of human foreskin melanocytes were performed as previously described [32] and melanocytes were cultured for a maximum of 10 passages in Ham's F10 (Flow) supplemented with 2% Ultroser-G synthetic serum (Gibco, Grand Island, NY), glutamate, penicillin, streptomycin, 0.1 mM IBMX (Sigma, St Louis, MO) and 16 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma).

Monoclonal antibodies

The following anti-integrin subunit mAbs were used. TS2/7 anti-α1 [15] (T-cell Sciences, Cambridge, MA); A1.43 anti-α2 [4,21] (Cell Diagnostica GmbH, Münster, Germany); 5E8 anti-α2 [45]; J143 anti-α3 [10]; GoH3 anti-α6 [33]; 4B4 anti-β1 [24] (Coulter Immunology, Hialeah, FL); 8A2 anti-β1 [22]; TS2/16 anti-β1 [15]; 3E1 anti-β4 [16] (Telios Pharmaceuticals Inc, San Diego, CA). As negative control in flowcytometry WT31 anti-CD3 [34,35] mAbs were used. As negative control in the adhesion inhibition assays W6/32 anti-MHC class I [3] mAbs were used. MHC class I is strongly expressed on all cell lines [41] and on cultured melanocytes (not shown).

Flowcytometry

Cells were harvested by short trypsinization of subconfluent monolayers. After washing with DMEM containing 10% FCS they were incubated with mAbs in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.02% azide for 30 min at 0°C. After washing three times with PBS/BSA/azide the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig (Dako, Glostrup, Denmark), or in the case of GoH3 mAbs, with FITC-conjugated rabbit anti-rat Ig (Dako). Analyses were performed on a Coulter Epics Elite (Coulter Electronics, Mijdrecht, The Netherlands).

Immunoprecipitation

Subconfluent monolayer cell cultures (75 cm^2) were labeled overnight at 37°C with 0.3 mCi [^{35}S]-methionine (Amersham, Houten, The Netherlands) in methionine-free medium (Flow) containing 10% dialysed FCS. The cells were washed 2 times with PBS and incubated with NP40 lysis buffer (0.5% NP40, 0.015 M NaCl, 0.01 M Tris pH 7.5, 1.0 mM phenyl-methyl-sulphonyl-fluoride and 4 μg/ml Aprotinin) at 4°C for 10 min. Subsequently the cells were scraped off the culture flask, repeatedly aspired into syringes and forced through needles with decreasing diameter. Glycoproteins were isolated from NP40 solubilized cell extracts by adsorption to concanavalin A (Con A) Sepharose (Pharmacia Inc, Uppsala, Sweden). Immunoprecipitations were performed as described before [20]. To compare the amount of glycoproteins in the various cell lines, equal numbers of counts of the Con A-bound fractions were used for immunoprecipitation.

Immunohistochemistry

After acetone fixation 4 μm frozen sections were incubated with mAbs for 1 h at room temperature. After washing with PBS, bound mAbs were visualized using peroxidase-conjugated rabbit anti-mouse Ig (Dako), or in the case of GoH3 mAbs, peroxidase-conjugated rabbit anti-rat Ig (Dako), and amino-ethyl carbazole (AEC). After counterstaining with hematoxylin the sections were mounted with Kaizers glycerin/gelatin.

Cell adhesion assay

Ln and CoIV, both isolated from Englebreth-Holm-Swarm mouse sarcoma cells, were purchased from Gibco. CoI isolated from rat tail was a gift from Dr E. Klein, Ulm, Germany. Fn isolated from human plasma, was purchased from Sigma. Polystyrene microtiterplates (96 wells, flatbottom, Greiner, Alphen a/d Rijn, The Netherlands) were coated by incubation overnight at 4°C with 50 μl of a solution containing 20 μg/ml of the appropriate adhesive protein in PBS. Unbound protein was removed by washing with PBS and free binding sites were blocked by incubation for 1 h at 37°C with DMEM containing 0.25% BSA. Cells were harvested by short trypsinization of subconfluent monolayers, washed with DMEM containing 0.25% BSA and labeled in a volume of approximately 100 μl with 50 μCi $Na_3{}^{51}CrO$ for 90 min at 37°C. Subsequently the cells were washed three times, diluted to a concentration of $1x10^5$ cells/ml in DMEM containing 0.25% BSA and seeded into the wells (5000 cells/well). Cells were allowed to attach for 30 min at 37°C in a 5% CO_2 atmosphere; non-adhered cells were removed by washing three times with DMEM containing 0.25% BSA; the attached cells were lysed with Triton- X-100 and radioactivity was measured in a gamma-counter. In adhesion inhibition/stimulation assays, labeled cells were incubated with the appropriate mAbs for 30 min at 4°C before seeding into the wells.

RESULTS

Metastatic behavior of human melanoma cell lines in nude mice

The rate of tumor take and the frequency of experimental and spontaneous metastasis of the human melanoma cell lines studied in nude mice has been described previously [41,42]. Although after subcutaneous inoculation all cell lines gave a good tumor take, only BLM and MV3 were found to be highly metastatic. IF6 and 530 did not metastasize at all and M14 and Mel57 were only poorly metastatic.

Adhesion to ECM components

In order to investigate whether the capacity to metastasize was reflected by the capacity to adhere to ECM components, we investigated adhesion to Ln, CoI, CoIV and Fn. Melanocytes, and the non- (IF6, 530) and poorly metastatic cells (M14, Mel57)

adhered weakly to Ln, CoI and CoIV whereas the highly metastatic BLM and MV3 melanoma cells adhered strongly to these ECM components (Fig 1). A different pattern was found for adhesion to Fn. Most cells exhibited strong binding to Fn and only 530 cells adhered weakly to Fn.

These results indicate that highly metastatic melanoma cells adhere to Ln and Co much stronger then non- or poorly metastatic cells.

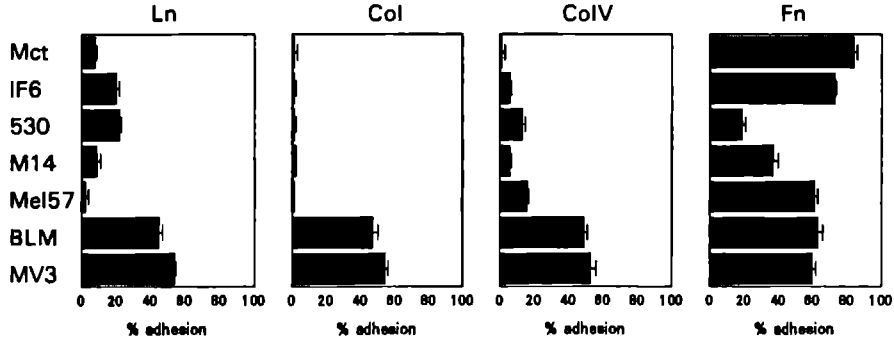


Figure 1. Adhesion of cultured melanocytes (MCT) and 6 human melanoma cell lines to Ln, CoI, CoIV, and Fn. Control adhesion to BSA was less than 5%. One experiment of 4 is shown. Means ± s.d. from triplicate wells are shown.

Expression of Ln/Co receptors

As adhesion to Ln and Co was strongly increased in highly metastatic cell lines, surface expression of integrins, known to mediate adhesion to Ln ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$) or Co ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$) was investigated by means of flowcytometry. Melanocytes and all 6 cell lines expressed $\beta1$ integrins (Fig 2). Beta-1 expression was high on melanocytes and the highly metastatic BLM and MV3 cells, and low on the other cell lines. Alpha-1 expression was low, both on melanocytes and on all cell lines. Notably, expression of $\alpha2$ and $\alpha6$ was high on the highly metastatic BLM and MV3 cells whereas melanocytes and all other cell lines showed no or only weak expression. The $\alpha3$ subunit was highly expressed on melanocytes and the highly metastatic BLM and MV3 cells, moderately on IF6 and Mel57 but not or hardly detectable on 530 and M14. No $\beta4$ expression was found on melanocytes or any of the cell lines even though 3E1 anti-$\beta4$ mAbs stained control cells (keratinocytes; not shown).

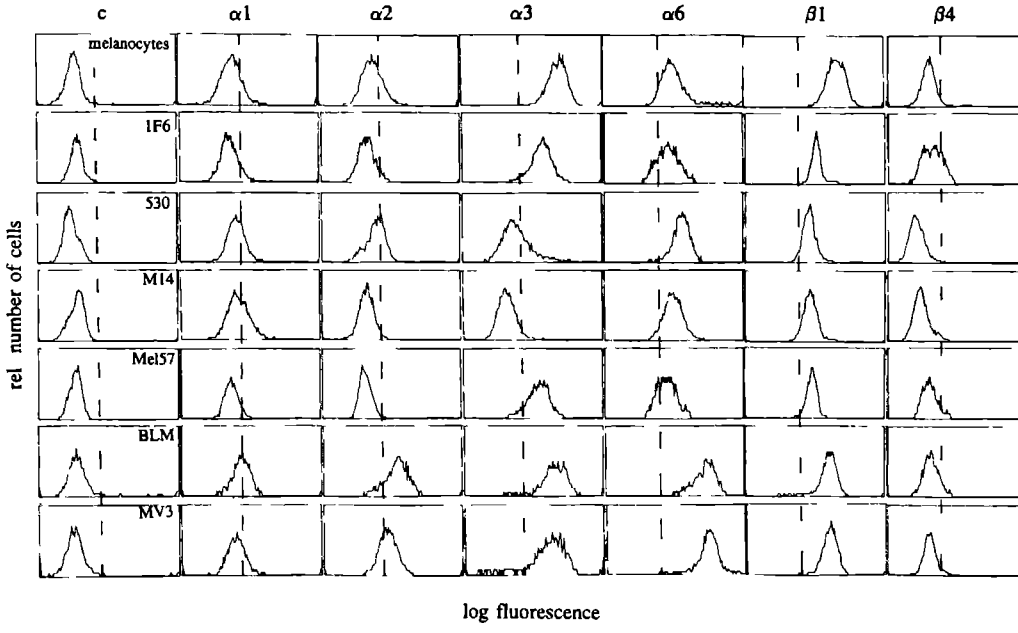


Figure 2. *Expression of Co/Ln receptors on cultured human melanocytes and 6 human melanoma cell lines. Cells were incubated with WT31 anti-CD3 control mAbs (c) or with TS2/7 anti-α1, A1.43 anti-α2, J143 anti-α3, GoH3 anti-α6, 4B4 anti-β1, or 3E1 anti-β4. The fluorescence intensity of 5000 cells was determined. One experiment of 2 is shown.*

To establish whether differences in cell surface expression were reflected by differences in the level of biosynthesis of integrin subunits, immunoprecipitation was performed on the glycoprotein fraction of [^{35}S]-methionine labeled melanoma cells. No α1 or β4 could be precipitated from any of the cell lines and α3 synthesis was detected in all cell lines except for 530 and M14 (Fig 3). Consistent with the surface expression data the level of synthesis of β1, α2, and α6 was increased in the highly metastatic BLM and MV3 cells compared with the other cell lines.

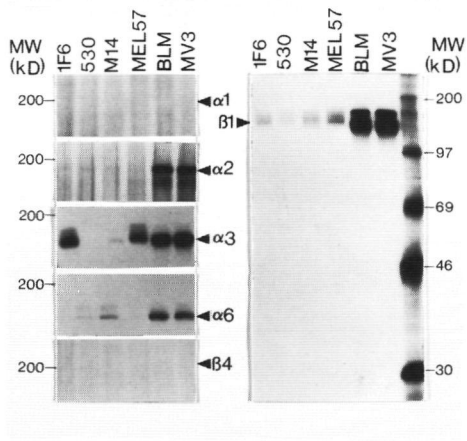


Figure 3. *Synthesis of Ln/Co receptors in 6 human melanoma cell lines. Glycoproteins were isolated from [^{35}S]-methionine-labeled cells with Con A-Sepharose. Equal numbers of counts were used for immunoprecipitation with TS2/7 anti-α1, A1.43 anti-α2, J143 anti-α3, GoH3 anti-α6, 4B4 anti-β1, or 3E1 anti-β4.*

Finally we determined whether increased expression of Ln/Co receptors was also found in situ. Immunohistochemistry was performed on subcutaneous xenograft lesions from the non-metastatic cell lines IF6 and 530 and from the highly metastatic cell lines BLM and MV3. Expression of α2 and α6 was only detected in lesions of the highly metastatic BLM and MV3 cells (Fig 4), similar to the results on cultured cells.

Thus, synthesis and expression of α6β1 and α2β1 in culture and in situ is strongly increased in highly metastatic melanoma cells compared to non- or poorly metastatic melanoma cells and normal melanocytes.

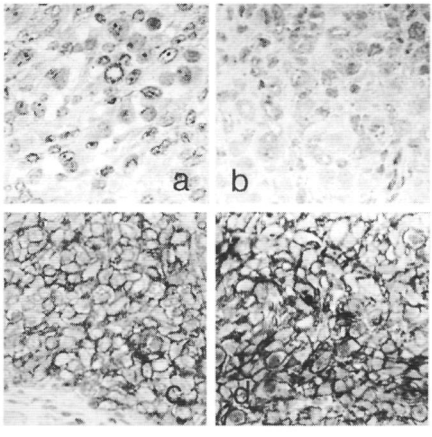


Figure 4. *Immunohistochemical staining of s.c. xenograft lesions from* ***A:*** *IF6 with A1.43 anti-α2,* ***B:*** *530 with GoH3 anti-α6,* ***C:*** *BLM with A1.43, or* ***D:*** *MV3 with GoH3.*

The role of α2β1 and α6β1 in adhesion to Ln and Co

To further demonstrate the role of α2β1 and α6β1 in adhesion to Ln, CoI, and CoIV, we performed antibody blocking studies. Since adhesion of normal melanocytes and the non- (IF6, 530) or poorly metastatic melanoma cells (M14 and Mel57) to Ln, CoI, or CoIV was absent or low (Fig 1), we only examined the highly metastatic BLM and MV3 cells. Adhesion of BLM and MV3 to Ln was inhibited for 60% by mAbs to α2, for 90%

by mAbs to $\alpha 6$, and the combination of these mAbs (Fig 5) or a mAb to $\beta 1$ (not shown) completely blocked adhesion of these cells to Ln (Fig 5). Adhesion to CoI and CoIV was almost completely inhibited by mAbs directed to $\alpha 2$ or $\beta 1$.

These results indicate that $\alpha 2\beta 1$ and $\alpha 6\beta 1$ are the major receptors for Co and Ln respectively on highly metastatic melanoma cells.

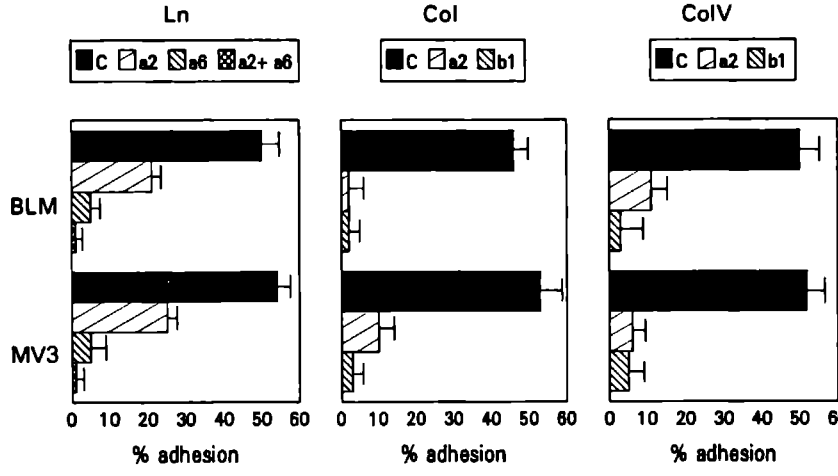


Figure 5. *Inhibition of adhesion to Ln/Co of BLM and MV3. Cells were allowed to adhere to wells coated with Ln, CoI, or CoIV in the absence or presence of W6/32 anti-MHC class I control mAbs (c) or 5E8 anti-$\alpha 2$, GoH3 anti-$\alpha 6$, 4B4 anti-$\beta 1$, or combinations. Adhesion to BSA was less then 5%. One experiment of 3 is shown. Means $\pm$ s.d. from triplicate wells are shown.*

Effect of stimulatory anti-$\beta 1$ mAbs on adhesion to Ln and Co

In order to determine whether differences in adhesion to Ln and Co between cells of varying metastatic capacities were not only due to differences in expression of Ln/Co receptors but also to the activation state of these receptors, we performed adhesion assays in the presence of 8A2 or TS2/16 anti-$\beta 1$ mAbs. Both mAbs have been described to stimulate integrin-mediated adhesion, probably by inducing a conformational change of the receptors. Following incubation with these mAbs, normal melanocytes and non- and poorly metastatic melanoma cells adhered strongly to Ln and CoI (Fig 6). No or only minor stimulatory effects were observed with the highly metastatic BLM and MV3 cells (not shown). The effect was inhibited by mAbs to $\alpha 6$ for Ln and by mAbs to $\alpha 2$ for CoI, demonstrating that $\alpha 6\beta 1$ and $\alpha 2\beta 1$ had been activated by the stimulatory $\beta 1$ mAbs.

From these results we conclude that $\alpha 6\beta 1$ and $\alpha 2\beta 1$ on normal melanocytes and non- and poorly metastatic melanoma cells are expressed in an inactive state whereas these receptors are constitutively active in highly metastatic melanoma cells.

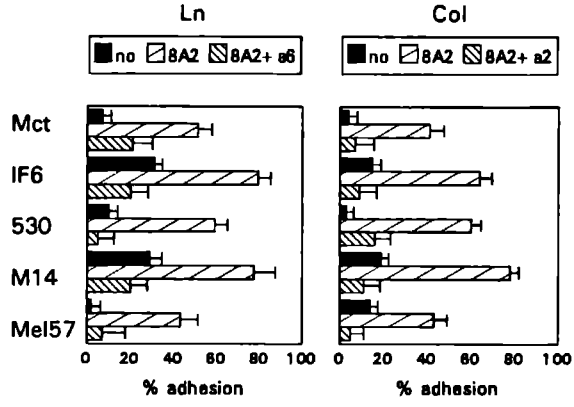


Figure 6. Stimulation of adhesion of cultured human melanocytes (MCT) and 4 human melanoma cell lines to Ln and Col with 8A2 anti-β1 mAbs and inhibition with G0H3 anti-α6 or 5E8 anti-α2 mAbs. Control adhesion to BSA was less then 5%. One experiment of 3 is shown. Means ± s.d. from triplicate wells are shown.

DISCUSSION

During metastasis, tumor cells interact at several sites with the ECM components of basal lamina and stroma. Therefore, we have compared the capacity of cultured human melanocytes and human melanoma cell lines with different metastatic capacities in nude mice to adhere to Ln, Co, and Fn.

No correlation between adhesion to Fn and the metastatic capacity of the cell lines was found. Since most cells bound well to Fn, a crucial role for Fn in the metastatic process would not be expected. This observation, confirms and extends the findings of a number of previous studies where no positive correlation between the capacity of cells to adhere to Fn and their tumorigenic and/or metastatic behavior was reported; a) chemical transformation of human osteosarcoma cells leads to changes in integrin expression but not of the $\alpha 3\beta 1$ and $\alpha 5\beta 1$ Fn receptors [8], b) mouse osteosarcoma cells with different metastatic capacities show similar interactions with Fn [2], and c) overexpression of the $\alpha 5\beta 1$ Fn receptor has even been demonstrated to result in loss of tumorigenicity in CHO

cells [11]. For melanoma cells, adhesion to Fn has been shown not to correlate with metastatic behavior in a nude mouse model [27,37] and cultured normal human melanocytes have been demonstrated to adhere very weakly to Ln and Co but strongly to Fn [12, this paper].

An important role for Ln in the metastatic behavior of melanoma cells has been demonstrated in several studies [2,36,37]. Our data confirm these findings and show that a) highly metastatic melanoma cells adhere strongly to Ln, b) this adhesion is mediated through $\alpha 6\beta 1$, and c) expression of the Ln receptor VLA-6 α-chain is markedly upregulated in highly metastatic human melanoma cells. Furthermore our results indicate that adhesion of human melanoma cells to Ln is not exclusively mediated through VLA-6 but also through $\alpha 2\beta 1$. Adhesion to CoI and CoIV has been demonstrated to be a property of various metastatic melanoma cell lines [7,23,30]. Our data confirm and extend these findings: the highly metastatic cells adhere strongly to CoI and CoIV whereas the non- or poorly metastatic cells and normal melanocytes adhere only weakly to these ECM components. Adhesion to both CoI and CoIV is mediated through $\alpha 2\beta 1$ which is strongly upregulated in highly metastatic cells. These results are in agreement with findings from Mortarini et al. [25] who reported high $\alpha 2$ and $\alpha 6$ expression on tumor cells cultured from human melanoma metastases but not on tumor cells cultured from primary melanomas. Finally, increased $\alpha 2$ expression in fresh human melanocytic lesions has been shown to correlate with malignant progression [4,21]. Our immunoprecipitation data indicate that elevated expression of $\alpha 2$ and $\alpha 6$ in highly metastatic cell lines is not only due to increased cell surface expression but also to increased biosynthesis. Furthermore, from our immunohistochemical data on subcutaneous xenograft lesions we conclude that the difference in expression of $\alpha 2\beta 1$ and $\alpha 6\beta 1$ between non- and highly metastatic cell lines is not only found in vitro but also in vivo.

It is well known that in order to interact with their ligands, integrins have to be in an active state [13,31,39]. For $\beta 1$ [38,44], $\beta 2$ [40], and $\beta 3$ integrins [14], mAbs have been described which induce a high avidity state of these receptors. Wayner and Kovach [44] have shown that U937 cells, T and B lymphoblastoid cells or PHA-stimulated T cell blasts require expression of the activated state of $\alpha 4\beta 1$ in order to bind the cell adhesion site in Fn, LDV, whereas A375 melanoma cells do not require such activation. These findings suggest that on certain melanoma cells integrins may be expressed in a constitutively active state. Our results extend these findings and show that non- or poorly metastatic melanoma cells require induction of a high avidity state of $\beta 1$ integrins in order to adhere to Ln and Co whereas highly metastatic cells do not. Even though only a very low amount of $\alpha 2\beta 1$ and $\alpha 6\beta 1$ is expressed on the non- or poorly metastatic cells, once these receptors are activated the cells adhere to Ln or Co as strong as the highly metastatic cells. This suggests that the number of receptors is less important than the state of activity they are in.

In conclusion, we show that $\alpha 2\beta 1$- and $\alpha 6\beta 1$-mediated adhesion to Co and Ln is strongly increased in highly metastatic human melanoma cell lines and that this adhesion can be regulated at the level of expression and activation of the $\alpha 2\beta 1$ and $\alpha 6\beta 1$ integrins.

ACKNOWLEDGEMENTS

We thank Drs. Richard Bankert, Eberhard Klein, Nicholas Kovach, Arnoud Sonnenberg, Tim Springer, and Wil Tax for kindly providing the antibodies, Drs. Daniëla Dressel and Eberhard Klein for their kind advice on the immunoprecipitation procedure, and Mrs. Ine Cornelissen for expert technical assistance. This work is supported by grant number NUKC 91-09 from the Dutch Cancer Society and the E.C. Concerted Action on melanoma progression.

REFERENCES

1. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: Association of the $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

2. Aresu O, Nicolò G, Allavena G, Melchiori A, Schmidt J, Kopp JB, d'Amore E, Chader GJ, Albini A. Invasive activity, spreading on and chemotactic response to laminin are properties of high but not low metastatic mouse osteosarcoma cells. Inv Metastasis 11, 2-13, 1991.

3. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens. New tools for genetic analysis. Cell 14, 9-20, 1978.

4. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36, 29-35, 1985.

5. Brüggen J, Sorg C, Macher E. Membrane-associated antigens of human malignant melanoma: Serological typing of cell lines using antisera from non human primates. Cancer Immunol Immunother 5, 53-68, 1978.

6. Chan BMC, Matsuura N, Takada Y, Zetter BR, Hemler ME. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. Science 251, 1600-1602, 1991.

7. Chelberg MK, Tsilibary EC, Hauser AR, McCarthy JB. Type IV collagen-mediated melanoma cell adhesion and migration: Involvement of multiple, distinct domains of the collagen molecule. Cancer Res 49, 4796-4802, 1989.

8. Dedhar S, Saulnier R. Alterations in integrin receptor expression on chemically transformed human cells: Specific enhancement of laminin and collagen receptor complexes. J Cell Biol 110, 481-489, 1990.

9. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.

10. Fradet Y, Cordon-Cardo C, Thomson T, Daly ME, Whitmore WFJr, Lloyd KO, Melamed MR, Old LJ. Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. Proc Natl Acad Sci USA 81, 224-228, 1984.

11. Giancotti FG, Ruoslahti E. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. Cell 60, 849-859, 1990.

12. Gilchrest BA, Albert LS, Karassik RL, Yaar M. Substrate influences human epidermal melanocyte attachment and spreading in vitro. In Vitr Cell Dev Biol 21, 114-120, 1985.
13. Ginsberg MH, Taylor L, Painter GG. The mechanism of thrombin-induced platelet factor 4 secretion. Blood 55, 661-668, 1980.
14. Gulino D, Ryckewaert JJ, Andrieux A, Rabiet MJ, Marguerie G. Identification of a monoclonal antibody against platelet GPIIb that interacts with a calcium-binding site and induces aggregation. J Biol Chem 265, 9575-9581, 1990.
15. Hemler ME, Sanchez-Madrid F, Flotte TJ, Krensky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL. Glycoproteins of 210,000 and 130,000 m.w. on activated T-cells: Cell distribution and antigenic relation to components on resting cells and T cell lines. J Immunol 132, 3011-3018, 1984.
16. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Basement membrane diversity detected by monoclonal antibodies. Differentiation 26, 49-54, 1984.
17. Hynes RO. Integrins: Versatility, modulation and signalling in cell adhesion. Cell 69, 11-25, 1992.
18. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmüller G De novo expression of intercellular adhesion molecule-1 in melanoma correlates with increased risk of metastasis. Proc Natl Acad Sci USA 86, 641-644, 1989.
19. Katano M, Saxton RE, Cochran AJ, Irie RF. Establishment of an ascitic human melanoma cell line that metastasizes to lung and liver in nude mice. J Cancer Res Clin Oncol 108, 197-203, 1984.
20. Klein CE. A transformation associated 130 KD cell surface glycoprotein is growth controlled in normal human cells. J Exp Med 167, 1684-1696, 1988.
21. Klein CE, Steinmeyer T, Kaufmann D, Weber L, Bröcker E-B Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96, 281-284, 1991.
22. Kovach NL, Carlos TM, Yee E, Harlan JM A monoclonal antibody to $\beta 1$ integrin (CD 29) stimulates VLA-dependent adherence of leukocytes to vascular cell adhesion molecule-1 (VCAM-1) J Cell Biol, 116, 499-509, 1992.
23. Kramer RH, Marks N. Identification of integrin collagen receptors on human melanoma cells. J Biol Chem 264, 4684-4688, 1989.
24. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.
25. Mortarini R, Anichini A, Parmiani G. Heterogeneity for integrin expression and cytokine-mediated VLA modulation can influence the adhesion of melanoma cells to extracellular matrix proteins. Int J Cancer 47, 551-559, 1991.
26. Murray JC, Liotta L, Rennard SI, Martin GR. Adhesion characteristics of murine metastatic and non-metastatic tumor cells in vitro. Cancer Res 40, 347-351, 1980.
27. Ormerod JE, Everet CA, Hart IA. Adhesion characteristics of human melanoma cell lines of varying metastatic potential. Int J Cancer 41, 150-154, 1988.
28. Plantefaber LC, Hynes RO. Changes in integrin receptors on oncogenically transformed cells. Cell 56, 281-290, 1989.
29. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. Nature 283, 139-146, 1980.
30. Ramos DM, Berston ED, Kramer RH. Analysis of integrin receptors for laminin and type IV collagen on metastatic B16 melanoma cells. Cancer Res 50, 728-734, 1990.
31. Shimizu Y, van Seventer GA, Horgan KJ, Shaw S. Regulated expression and binding of three VLA ($\beta 1$) integrin receptors on T cells. Nature 345, 250-253, 1990.
32. Smit NPM, Westerhof W, Asghar SS, Pavel S, Siddiqui AH Large scale cultivation of human melanocytes using collagen-coated sephadex beads (cytodex 3). J Invest Dermatol 92, 18-21, 1989.

33. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. J Biol Chem 264, 13745-13750, 1987.

34. Spits H, Borst J, Tax WJM, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 135, 1922-1928, 1985.

35. Tax WJM, Willems HW, Reekers PPM, Capel PJA, Koene RAP. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells Nature 304, 445-447, 1983.

36. Terranova VP, Liotta LA, Russo RG, Martin GR. Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res 42, 2265-2269, 1982.

37. Terranova VP, Williams JE, Liotta LA, Martin GR. Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. Science 226, 982-984, 1984.

38. Van de Wiel-van Kemenade E, van Kooyk Y, de Boer AJ, Huijbens RJF, Weder P, van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

39. Van Kooyk Y, van de Wiel-van Kemenade P, Weder P, Kuijpers TW, Figdor CG Enhancement of LFA-1 mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature 342, 811-813, 1989.

40. Van Kooyk Y, Weder P, Hogervorst F, Verhoeven AJ, van Seventer G, te Velde AA, Borst J, Keizer G, Figdor CG. Activation of LFA-1 through a Ca^{2+}-dependent epitope stimulates lymphocyte adhesion. J Cell Biol 112, 345-354, 1991.

41. Van Muijen GNP, Cornelissen LMHA, Jansen CFJ, Figdor CG, Johnson JP, Bröcker E-B, Ruiter DJ. Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin Expl Metast 9, 259-272, 1991.

42. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ. Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. Int J Cancer 48, 85-91, 1991.

43. Versteeg R, Noordermeer IA, Krüsse-Wolters M, Ruiter DJ, Schrier PI. C-myc down-regulates class I HLA expression in human melanomas. EMBO J 7, 1023-1029, 1988.

44. Wayner EA, Kovach NL. Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V-region of fibronectin. J Cell Biol 116, 489-497, 1992.

45. Zylstra S, Chen FA, Ghosh SK, Repasky EA, Rao U, Takita H, Bankert RB Membrane associated glycoprotein (gp160) identified on human lung tumor by a monoclonal antibody. Cancer Res 48, 2768-2773, 1986.

Alpha-v integrins in human melanoma: gain of αvβ3 and loss of αvβ5 are related to tumor progression in situ but not to metastatic capacity of cell lines

Int J Cancer **61**, 491-496, 1995

Alpha-v integrins in human melanoma: gain of αvβ3 and loss of αvβ5 are related to tumor progression in situ but not to metastatic capacity of cell lines

Erik HJ Danen, Kees FJ Jansen, Annemieke A Van Kraats, Ine MHA Cornelissen, Dirk J Ruiter, and Goos NP van Muijen

Department of Pathology, University Hospital, Nijmegen, The Netherlands

We investigated the expression of αv-integrins in different stages of human cutaneous melanocytic tumor progression. We observed that αvβ5 was the αv-integrin expressed in all common nevocellular nevi, in 78% of dysplastic nevi, in 63% of early primary melanomas, in 43% of advanced primary melanomas, and in 33% of melanoma metastases. Hence, loss of αvβ5 expression was related to melanocytic tumor progression. In line with earlier reports, αvβ3 was exclusively detected in advanced primary melanomas and metastases (24% and 50% respectively). Staining with anti-αv mAbs on lesions where both αvβ3 and αvβ5 were absent, showed that alternative αv-integrins were expressed in advanced primary melanomas and metastases. By FACS analysis, we determined expression of αvβ5 and αvβ3 on 4 human melanoma cell lines with different metastatic capacities after subcutaneous inoculation into nude mice. One of the non-metastatic and both highly metastatic cell lines expressed αvβ5 at their surface. Surprisingly, αvβ3 was detected exclusively on the non-metastatic cell lines. Absence of αvβ3 in the highly metastatic cell lines was confirmed by lack of immunoprecipitation from [^{35}S]-methionine labeled cells and by absence of immunohistochemical staining on primary and metastatic xenograft lesions. Our findings indicate that αvβ5 expression is often lost in advanced stages of melanocytic tumor progression in situ while αvβ3 is acquired, but that decrease of αvβ5 and increase of αvβ3 expression is not necessarily related to the metastatic behavior of human melanoma cells in nude mice.

INTRODUCTION

The extracellular matrix (ECM) regulates a number of cellular processes and integrins link the ECM to structural elements in the cell and play a role as signaling receptors [9]. Therefore, it is likely that integrins can mediate ECM control of cell growth, migration, and invasion. These processes play an important role in tumorigenicity and metastasis formation and integrins have indeed been shown to be involved in both phenomena [10].

In human melanoma, integrins have been demonstrated to be involved in tumor growth and metastatic spread [14]. For melanocytic tumor progression in situ, changes in the expression of several integrins have been reported, including acquired expression of $\alpha v\beta 3$ in the vertical growth phase of primary melanomas and in metastases [2]. The αv subunit of this integrin, however, can be associated with several different β subunits in melanoma cells in vitro [12] and is expressed in all stages of melanocytic tumor progression in situ [4]. In this study we have investigated expression of αv-integrins in cutaneous melanocytic lesions and in a panel of human melanoma cell lines with different metastatic capacities in nude mice.

MATERIAL AND METHODS

Lesions

Lesions were obtained from patients at the University Hospital, Nijmegen, The Netherlands and at the University Hospital, Würzburg, Germany. Based on histopathologic examination of paraffin sections, lesions were divided into five classes: common nevocellular nevus (NN) (n=19), dysplastic [7] (atypical) nevus (DN) (n=9), early primary melanoma (tumor thickness$\leq$1.5 mm; ePM) (n=8), advanced primary melanoma (tumor thickness$>$1.5 mm; aPM) (n=21) and melanoma metastasis (MM) (n=24). Representative samples were snap frozen in liquid nitrogen and stored at -80°C until sectioning.

Cell lines and culture conditions

The melanoma cell lines used, included: IF6 [19], 530 [21], BLM [19], MV3 [20] and Mel57 [19]. All cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM; Flow laboratories, Irvine, UK) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin.

Monoclonal antibodies

Anti-integrin mAbs were 4B4 anti-$\beta 1$ [13] (Coulter, Hialeah, FL), LM142 anti-αv and LM609 anti-$\alpha v\beta 3$ [3] (Dr. David Cheresh, La Jolla, CA), 13C2 anti-αv and 23C6 anti-$\alpha v\beta 3$ [6] (Dr. Michael Horton, London, UK), and P1F6 anti-$\alpha v\beta 5$ [22] (Telios, San

Diego, CA). In flowcytometry, WT31 anti-CD3 mAbs [17] (Dr. Wil Tax, Nijmegen, The Netherlands) were used as a negative control. NKI-beteb anti-gp100 [1] (Dr. Carl Figdor, Nijmegen, The Netherlands) was used to identify melanocytic cells in human lesions.

Immunohistochemistry

Identical procedures were used for immunohistochemistry on frozen sections of human melanocytic lesions and melanoma cell line xenograft lesions. Four μm frozen sections were fixed in acetone for 10 min and incubated at room temperature with mAbs for 1 h. After washing with phosphate buffered saline (PBS) bound mAbs were visualized using the peroxidase-based Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole as substrate. After counterstaining with Mayer's hematoxylin sections were mounted with Kaisers glycerin/gelatin (Merck, Darmstadt, Germany).

Melanocytic cells were identified in HE stained sections and by staining with NKI-beteb mAbs. The percentage of stained melanocytic cells was estimated as 0, 1-25%, 51-75%, or 76-100%. Slides were read independently by two observers. Discrepancies exceeding more than one percentage class were found in less than 10% of the cases. These cases were revaluated jointly until consensus was reached. Logistic regression was used to determine a correlation between antigen expression and tumor progression.

Flow cytometry

Cells were harvested by short trypsinization of subconfluent monolayers and suspended in DMEM/10% FCS. After washing with PBS containing 0.5% BSA and 0.02% azide, they were incubated with mAbs in PBS/BSA/azide for 30 min at 4°C, washed with PBS/BSA/azide, and incubated with fluorescein-isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). Analyses were performed on an Epics Elite flowcytometer (Coulter, Mijdrecht, The Netherlands).

Immunoprecipitation

Immunoprecipitations were performed as described before [5]. In short, cells were labeled overnight with 0.3 mCi [^{35}S]-methionine (Amersham, Houten, The Netherlands), washed and lysed with 0.5% NP40 lysis buffer. Glycoproteins were isolated from NP40 solubilized cell extracts by adsorption to concanavalin A (Con A) Sepharose (Pharmacia, Uppsala, Sweden). To compare the amount of glycoproteins in the different cell lines, equal numbers of counts of the Con A-bound fractions were used for immunoprecipitation. MAbs, rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and Prot A beads (Pharmacia) were subsequently added and the volume was adjusted to 1 ml with 0.5% NP40. Samples were tumbled overnight at 4°C. Beads were washed 3 times with 0.5% NP40, 5 times with 0.5% NP40/0.1% SDS, resuspended in sample buffer

containing 2-mercaptoethanol, boiled, and run on SDS-PAGE.

RESULTS

Expression of αvβ5 in situ

In order to investigate which αv-integrins are expressed in benign melanocytic lesions and to see whether other αv-integrins besides αvβ3 are expressed in malignant melanoma we stained a series of NN, DN, ePM, aPM and MM with P1F6 anti-αvβ5 mAbs. Besides staining of fibroblast-like cells in stroma of all lesions, staining of melanocytic cells was found in 100% of NN (19/19), in 78% of DN (7/9), in 63% of ePM (5/8), in 43% of aPM (9/21) and in 33% of MM (8/24) (Figs 1,2). Hence, loss of expression of αvβ5 was related to tumor progression (p=0.0001). In the lesions that were positive for αvβ5, a variable heterogeneous staining pattern was observed with 25-100% positive melanocytic cells.

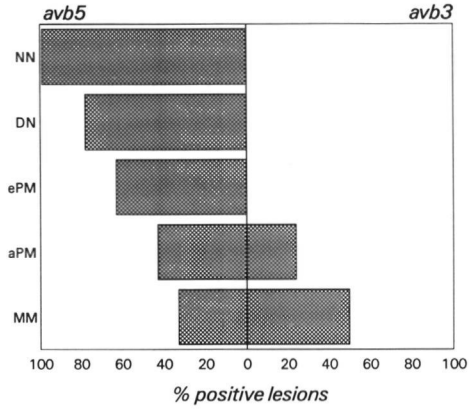


Figure 1: *Expression of αvβ5 and αvβ3 in different stages of human melanocytic tumor progression. 19 NN, 9 DN, 8 ePM, 21 aPM, and 24 MM were stained with P1F6 anti-αvβ5 and 23C6 and LM609 anti-αvβ3 mAbs. Percentage of lesions whith positive melanocytic cells are indicated. In the lesions that were positive, 25-100% of melanocytic cells stained.*

Expression of other αv-integrins in situ

In contrast to αvβ5, staining for αvβ3 was absent in NN, DN and ePM whereas 24% of aPM (5/21) and 50% of MM (12/24) were positive, indicating that αvβ3 emerged in aPM and MM (p=0.0001) (Figs 1,2). Incubation with 23C6 or LM609 anti-αvβ3 mAbs gave similar results. In all lesions staining of bloodvessels was observed. In 2 DN, 4 ePM, 10 aPM and 6 MM neither αvβ3 nor αvβ5 could be detected and we incubated these lesions with 13C2 and LM142 anti-αv mAbs. Staining was negative for the 2 DN and 4 ePM lesions, whereas 5/10 aPM and 5/6 MM lesions were positive indicating that other αv integrins were expressed (not shown). Incubation with 4B4 anti-β1 mAbs resulted in staining of all melanocytic cells in all lesions (not shown), indicating that αvβ1 may possibly be the αv integrin expressed in aPM and MM lesions.

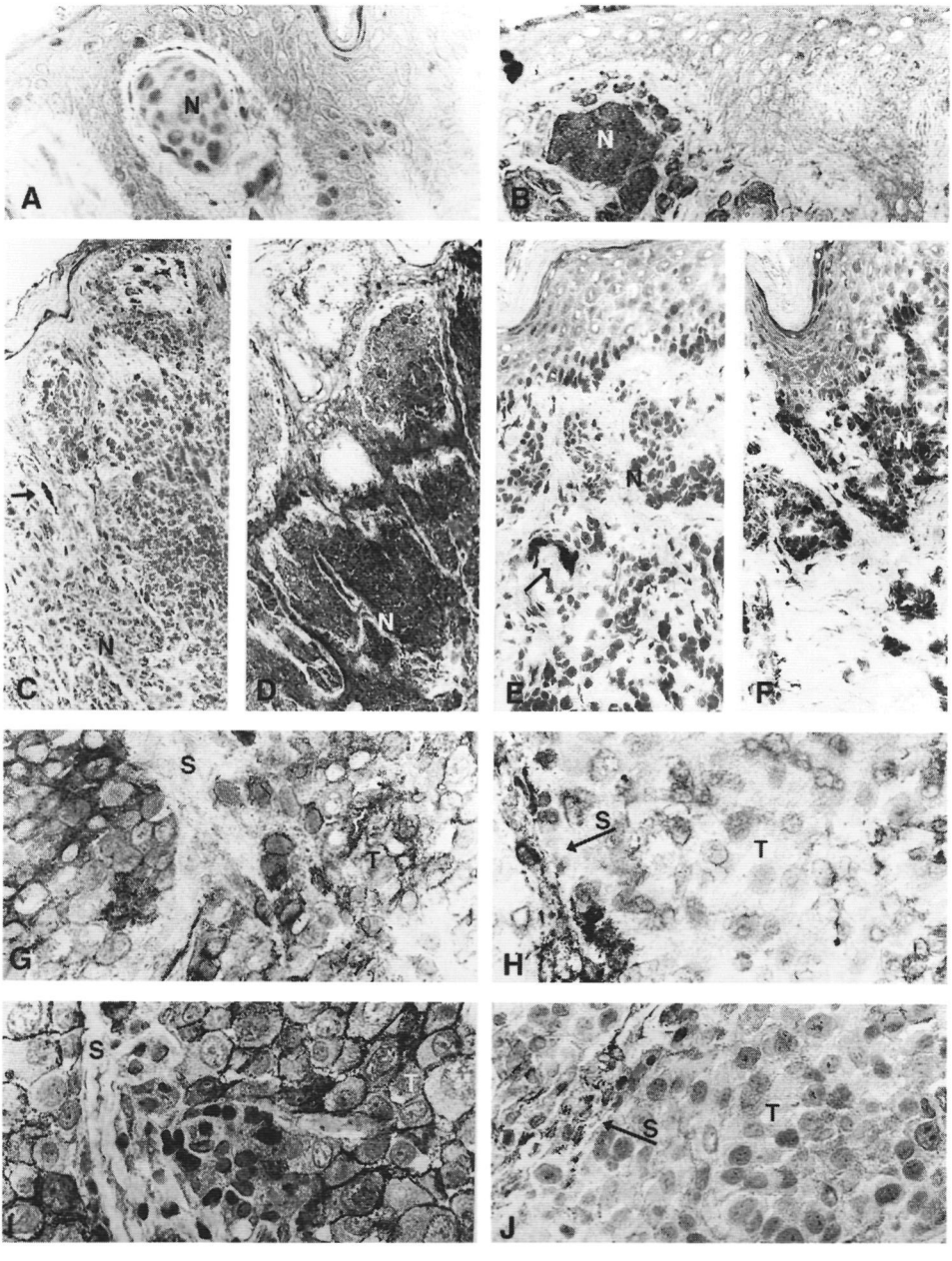

A
N
B
N
C
N
D
N
E
N
F
N
G
S
T
H
S
T
J
S
T

Figure 2: *Microphotographs of melanocytic lesions stained with anti-αv-integrin mAbs. Melanocytic lesions were stained with 23C6 and LM609 anti-αvβ3 or P1F6 anti-αvβ5 mAbs. Arrowheads indicate nevus (A-D) or melanoma (E-J) cells.* ***A&B:*** *NN negative for αvβ3 (A) and positive for αvβ5 (B).* ***C&D:*** *DN negative for αvβ3 (C; arrow=positive bloodvessel) and positive for αvβ5 (D).* ***E&F:*** *ePM negative for αvβ3 (E; arrow=positive bloodvessel) and positive for αvβ5 (F).* ***G&H:*** *aPM positive for αvβ3 (G) and negative for αvβ5 (H) (s=stroma cells).* ***I&J:*** *MM positive for αvβ3 (I) and negative for αvβ5 (J) (s=stroma cells). Bars are 20μm. (see opposite page).*

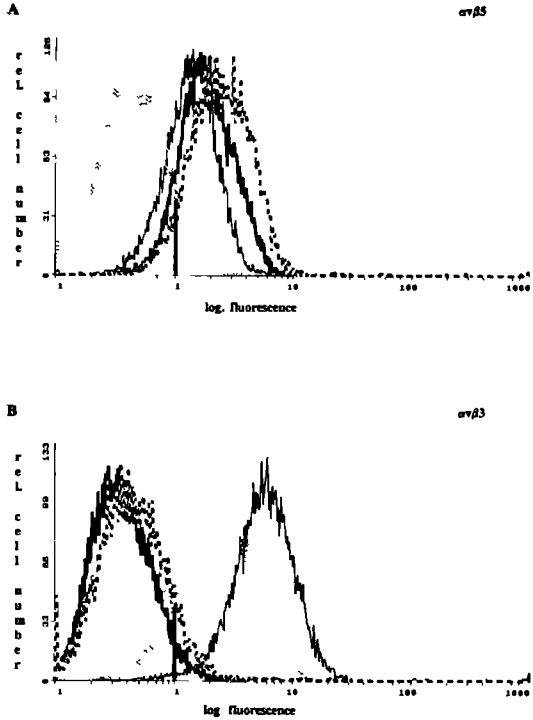


Figure 3: *Surface expression of αv-integrins on human melanoma cell lines. IF6 (thin line), 530 (thin dotted line), BLM (thick line) and MV3 (thick dotted line) were incubated with P1F6 anti-αvβ5* ***(A)*** *or LM609 anti-αvβ3 mAbs* ***(B)*** *followed by FITC-labeled second antibodies. Fluorescence was measured on an Epics Elite flowcytometer. The vertical line indicates the gate set with control anti-CD3 mAbs. Results with 23C6 mAbs were identical to those with LM609.*

Expression of αvβ5 and αvβ3 in human melanoma cell lines

We next examined whether decreased expression of αvβ5 and increased expression of

αvβ3 also correlated with the metastatic potential of cultured human melanoma cells. Therefore we used a panel of 4 human melanoma cell lines. After subcutaneous inoculation into nude mice all 4 cell lines have been shown to be tumorigenic but IF6 and 530 do not or only in a very low percentage of mice give rise to metastases whereas BLM and MV3 very frequently metastasize [19,20]. FACS analysis showed that comparable levels of αvβ5 were expressed on IF6, BLM and MV3 whereas no αvβ5 could be detected on 530 cells. (Fig 3a). Surprisingly, no αvβ3 was detected on the highly metastatic cell lines BLM and MV3 whereas the non-metastatic cell lines IF6 and 530 expressed αvβ3 at their surface (Fig 3b). Hence, the relation of decreased αvβ5- and increased αvβ3 expression with melanocytic tumor progression in situ, was not paralleled by a relation with the metastatic capacity of human melanoma cells in nude mice.

In order to investigate whether the absence of αvβ3 from the surface of BLM and MV3 cells was reflected by a lack of biosynthesis of αvβ3 in these cells, immunoprecipitations were performed on [^{35}S]-methionine labeled cells. Consistent with the surface expression data, synthesis of αvβ3 was extremely low for the highly metastatic BLM and MV3 cells. For the non-metastatic IF6 and 530 cells, a clear 125 kDa band corresponding to αv and a 105 kDa band corresponding to β3 were detected, whereas these bands were barely visible for BLM and MV3 cells (Fig 4). The 90 kDa and 150 kDa bands were non-specific since they could be detected even after incubation with normal mouse serum (NMS).

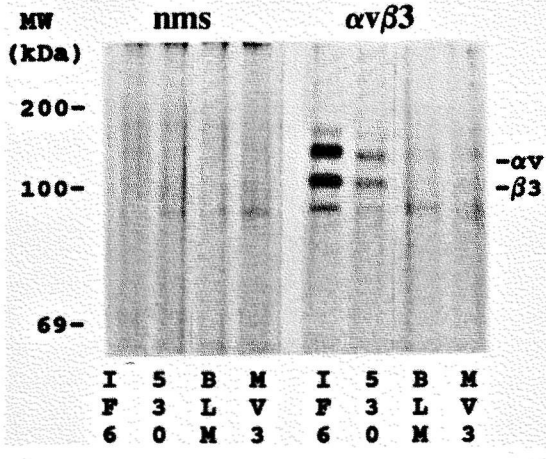


Figure 4: Biosynthesis of αvβ3 in human melanoma cell lines. IF6, 530, BLM and MV3 were metabolically labeled with [^{35}S]-methionine, lysed, and glycoproteins were isolated on Con A-Sepharose. Equal numbers of Con A-bound counts were used for immuno-precipitation with 23C6 anti-αvβ3 mAbs or NMS as a negative control. Identical results were obtained with LM609 mAbs.

Expression of αvβ3 in xenograft lesions

In order to exclude the possibility that absence of αvβ3 in the highly metastatic cell lines in vitro was due to culture conditions, we stained xenograft lesions of these cell

lines with anti-$\alpha v\beta 3$ mAbs. No $\alpha v\beta 3$ was detected in primary tumors or metastases of BLM and MV3 cells, whereas control anti-$\beta 1$ mAbs stained all tumor cells (Fig 5). For IF6 and 530 cells that expressed $\alpha v\beta 3$ in vitro, we could not detect $\alpha v\beta 3$ in xenograft lesions (not shown), suggesting that the level of expression was too low to detect immunohistochemically or, alternatively, culturing the cells may influence the expression of $\alpha v\beta 3$. Therefore, as a positive control, we stained subcutaneous xenograft lesions of Mel57 melanoma cells that strongly express $\alpha v\beta 3$ in vitro (our unpublished data). As shown in figure 5, Mel57 melanoma cells stained strongly with LM609 mAbs in xenograft lesions. Hence, in line with the findings in vitro, subcutaneous tumors and lung metastases in nude mice of the highly metastatic human melanoma cell lines BLM and MV3 do not show $\alpha v\beta 3$ expression.

DISCUSSION

Cutaneous melanoma is characterized by proliferative and invasive growth in the dermis and is often followed by widespread metastasis. Interactions of tumor cells with the ECM, which are mainly mediated by integrins [9], are thought to play an important role in the malignant behavior of melanoma [14] and other human tumors [10]. In the present study, we investigated the expression of αv-integrins in human melanocytic tumor progression in situ, and in a panel of human melanoma cell lines with different metastatic capacities after subcutaneous inoculation into nude mice.

Acquired expression of $\alpha v\beta 3$ in the vertical growth phase of primary melanomas and in melanoma metastases has been reported [2] whereas the αv subunit is expressed in all stages of melanocytic tumor progression [4]. In vitro, melanoma cells have been shown to express $\alpha v\beta 3$, $\alpha v\beta 1$ [12] and $\alpha v\beta 5$ [22]. In this study we have investigated which of the αv integrins are expressed in situ in nevi and in primary melanomas and metastases. Our data concerning $\alpha v\beta 3$ expression confirm the findings from previous studies [2,4] and show that $\alpha v\beta 3$ emerges in aPM and MM. Regarding $\alpha v\beta 5$ we find that expression is often lost with melanocytic tumor progression. Since no mAbs have yet been generated recognizing the $\alpha v\beta 1$ complex we could not investigate expression of this integrin. For the lesions with $\alpha v\beta 3$ and/or $\alpha v\beta 5$ expression we cannot exclude that other αv integrins are expressed as well. For those lesions where neither $\alpha v\beta 3$ nor $\alpha v\beta 5$ was detected we investigated whether alternative αv-integrins are expressed. The fact that alternative αv integrins were not detected in DN or ePM whereas a number PM and MM did express other αv integrins, suggests that additional αv integrins may emerge in aPM and MM besides $\alpha v\beta 3$. Thus, in NN, DN and ePM $\alpha v\beta 5$ can be expressed whereas in aPM and MM $\alpha v\beta 5$, $\alpha v\beta 3$ and/or other αv integrins can be expressed. Since all lesions were $\beta 1$ positive, and since $\alpha v\beta 1$ can be expressed by melanoma cells in vitro [12], $\alpha v\beta 1$ may be the alternative αv-integrin emerging.

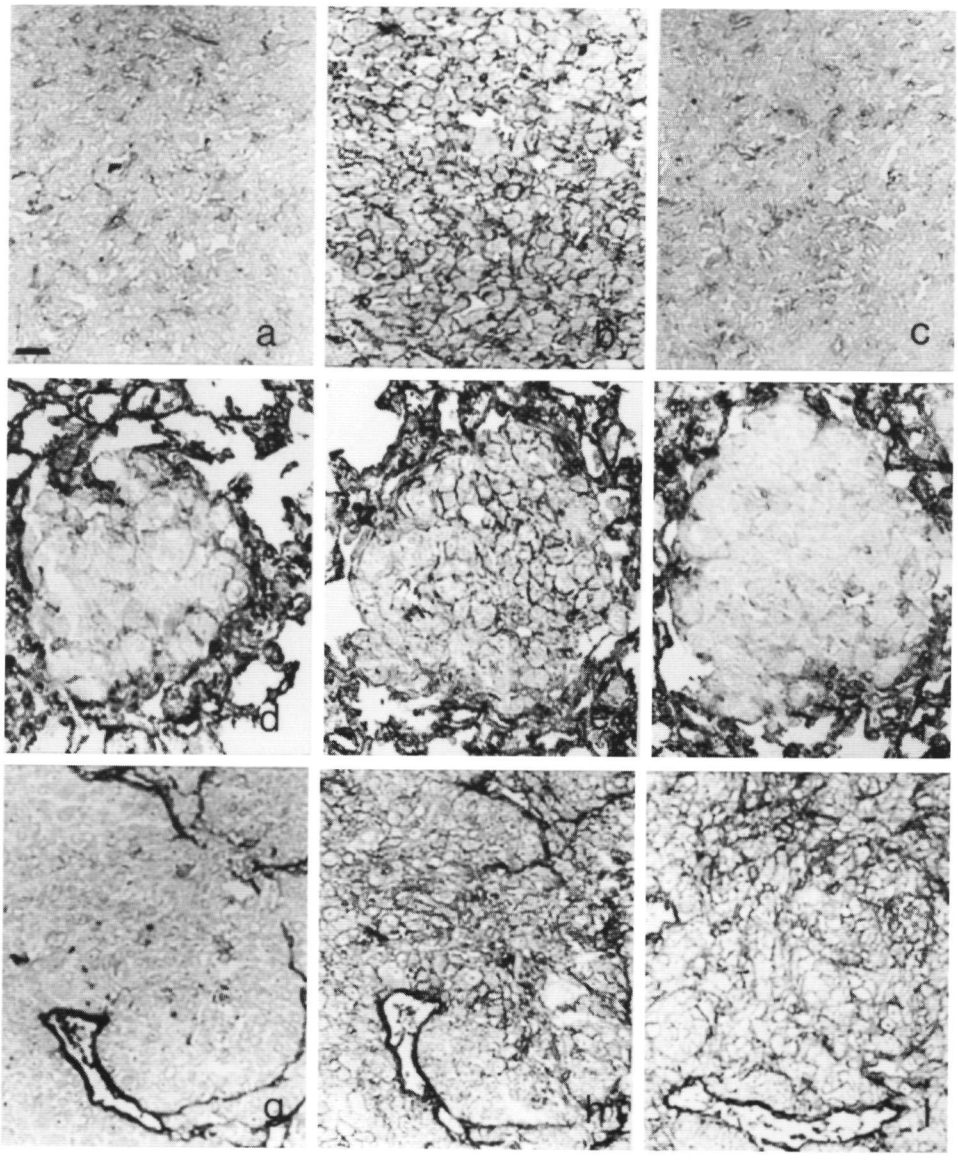


Figure 5: *Expression of αvβ3 in xenograft lesions. Frozen sections from MV3 subcutaneous tumor* ***(A,B,C)****, MV3 lung metastasis* ***(D,E,F)****, and Mel57 subcutaneous tumor* ***(G,H,I)****, were stained either in the absence of primary mAbs* ***(A,D,G)****, with 4B4 anti-β1* ***(B,E,H)****, or with LM609 anti-αvβ3 mAbs* ***(C,F,I)****. Results for BLM were identical to those shown for MV3. Bar is 20μm.*

The fact that αvβ5 expression is lost in most MM and that expression of αvβ3

emerges may have functional consequences for the melanoma cells. The ligand binding specificity of $\alpha v\beta 5$ seems to be restricted to vitronectin whereas $\alpha v\beta 3$ recognizes multiple ligands including vitronectin and fibronectin [16]. In addition, $\alpha v\beta 5$ and $\alpha v\beta 3$ promote distinct cellular responses to vitronectin in vitro [11]. We have recently shown that for melanomas originating from the uvea, $\alpha v\beta 3$ is absent in all primary lesions including those of the aggressive type, and that $\alpha v\beta 5$ is the αv integrin expressed [18]. This may indicate that the microenvironment of the melanoma cells is important in determining which αv-integrins are expressed. The fact that a role in proliferation [8] and invasion [15] of melanoma cells has been attributed to $\alpha v\beta 3$, seems to be in line with the emergence of $\alpha v\beta 3$ in aPM and MM. In our panel of human melanoma cell lines, IF6 and 530 express $\alpha v\beta 3$ in vitro but no $\alpha v\beta 3$ can be detected in the primary xenograft tumors, and these tumors grow slowly. On the other hand, BLM and MV3 tumors lack $\alpha v\beta 3$, and these tumors grow fast. All 4 cell lines develop tumors upon s.c. inoculation into nude mice [19,20]. This suggests that $\alpha v\beta 3$ is not necessarily involved in melanoma tumor growth. Furthermore, the fact that for the highly metastatic cell lines, $\alpha v\beta 3$ expression is absent in vitro, in subcutaneous xenograft lesions, and in lung metastases, suggests that they use alternative integrins for metastasizing in nude mice.

In conclusion, we show that $\alpha v\beta 5$ is often lost in advanced stages of melanocytic tumor progression in situ while $\alpha v\beta 3$ emerges, but that decrease of $\alpha v\beta 5$ and increase of $\alpha v\beta 3$ is not necessarily related to the metastatic potential of human melanoma cell lines in nude mice.

ACKNOWLEDGEMENTS

We thank Drs. David Cheresh, Carl Figdor, Michael Horton, and Wil Tax for generously providing antibodies, Dr. Eva-Bettina Bröcker for kindly providing some of the lesions, and Dr. Bep van 't Hof-Grootenboer for statistical analyses. This work was supported by grant NUKC 91-09 from the Dutch Cancer Society.

REFERENCES

1. Adema GJ, De Boer AJ, Van 't Hullenaar R, Denijn, M, Ruiter DJ, Vogel AM, Figdor CG. Melanocyte lineage specific antigens recognized by monoclonal antibodies NKI-beteb, HMB50, and HMB45 are encoded by a single cDNA. Am J Pathol, 143, 1597-1585, 1993.

2. Albelda SM, Mette SA, Elder, DE, Stewart R, Damjanovich L, Herlyn M, Buck C Integrin distribution in malignant melanoma: Association of the $\beta 3$ subunit with tumor progression Cancer Res, 50, 6757-6764, 1990.

3. Cheresh DA, Spiro RC. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J Biol

Chem, 262, 17703-17711, 1987.

4. Danen EHJ, Ten Berge PJM, Van Muijen GNP, Van 't Hof-Grootenboer B, Bröcker EB, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor during melanoma progression. Histopathol, 24, 249-256, 1994.

5. Danen EHJ, Van Muijen GNP, Van de Wiel-Van Kemenade P, Jansen CFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells Int J Cancer, 54, 315-321, 1993.

6. Davies J, Warwick J, Totty N, Philip R, Helfrich M, Horton M. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J Cell Biol, 109, 1817-1826, 1989.

7. De Wit PEJ, Van 't Hof-Grootenboer B, Ruiter DJ, Bondi R, Bröcker EB, Cesarini JP, Hastrup N, Hou-Jensen K, Mackie RM, Scheffer E, Suter L, Urso C Validity of the histopathological criteria used for diagnosing dysplastic naevi Eur J Cancer, 29A, 831-839, 1993.

8. Felding-Habermann B, Mueller BM, Romerdahl C, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest, 89, 2018-2022, 1992.

9. Hynes RO. Integrins: versatility, modulation and signalling in cell adhesion Cell, 69, 11-25, 1992.

10. Juliano RL, Varner JA. Adhesion molecules in cancer: the role of integrins. Curr Opin Cell Biol, 5, 812-818, 1993.

11. Leavesly DI, Ferguson GD, Wayner EA, Cheresh DA. Requirement of the integrin $\beta 3$ subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen J Cell Biol, 117, 1101-1107, 1992.

12. Marshall JF, Nesbitt SA, Helfrich MH, Horton MA, Polankova K, Hart IR Integrin expression in human melanoma cell lines: heterogeneity of vitronectin receptor composition and function Int J Cancer, 49, 924-931, 1991.

13. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset J Immunol, 134, 3762-3769, 1985.

14. Mortarini R, Anichini A. From adhesion to signalling: roles of integrins in the biology of human melanoma. Melanoma Res, 3, 87-97, 1993

15. Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the $\alpha v\beta 3$ integrin in human melanoma cell invasion Proc Natl Acad Sci (USA), 89, 1557-1561, 1992.

16. Smith JW, Vestal DJ, Irwin SV, Burke TA, Cheresh DA Purification and functional characterization of integrin $\alpha v\beta 5$: an adhesion receptor for vitronectin J Biol Chem, 265, 11008-11013, 1990.

17. Tax WJM, Willems HW, Reekers PPM, Capel PJA, Koene RAP Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature, 304, 445-447, 1983

18. Ten Berge PJM, Danen EHJ, Van Muijen GNP, Jager MJ, Ruiter DJ Integrin expression in uveal melanoma differs from cutaneous melanoma. Invest Ophtalmol Vis Sci, 34, 3635-3640, 1993

19. Van Muijen GNP, Cornelissen IMHA, Jansen CFJ, Figdor CG, Johnson JP, Bröcker EB, Ruiter DJ. Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin Expl Metast, 9, 259-272, 1991

20. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. Int. J. Cancer, 48, 85-91, 1991.

21. Versteeg R, Noordermeer IA, Krüsse-Wolters M, Ruiter DJ, Schrier PI. C-myc down-regulates class I HLA expression in human melanomas EMBO J., 7, 1023-1029, 1988.

22. Wayner EA, Orlando RA, Cheresh DA Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J Cell Biol, 113, 919-929, 1991.

Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin $\alpha 5\beta 1$

J Biol Chem **270**, 21612-21618, 1995

Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin $\alpha5\beta1$

Erik HJ Danen[1], Shin-ichi Aota[2], Annemieke A van Kraats[1], Kenneth M Yamada[2], Dirk J Ruiter[1], and Goos NP van Muijen[1]

[1]*Department of Pathology, University Hospital, Nijmegen, The Netherlands, and*
[2]*Laboratory of Developmental Biology, NIDR, NIH, Bethesda, MD*

We investigated the influence of the activation state of integrin $\alpha5\beta1$ on its dependence on the PHSRN synergy site for binding to RGD in fibronectin. K562 and MV3 cells lacked $\alpha v\beta3$ expression and adhered to fibronectin through $\alpha5\beta1$. Mel57 cells adhered through $\alpha v\beta3$ and $\alpha5\beta1$. K562 showed no adhesion to mono- or polymeric GRGDSP peptides and MV3 adhered only weakly to the polymeric peptide. RGD-containing peptides promoted Mel57 adhesion to the same extent as fibronectin. Soluble GRGDSP blocked Mel57 adhesion to fibronectin but had no effect on adhesion of K562 and MV3. A recombinant fibronectin polypeptide containing 3Fn6-3Fn10, and a mutated polypeptide lacking the synergy site, were equally effective in promoting Mel57 adhesion. For K562 and MV3 the mutated polypeptide was not or poorly active compared to the control polypeptide. Expression of $\alpha v\beta3$ in MV3 cells induced strong adhesion to the mutated polypeptide. TS2/16 or 8A2 stimulatory $\beta1$-integrin antibodies or Mn^{2+} induced $\alpha5\beta1$-mediated adhesion of K562 and MV3 to GRGDSP. In the presence of TS2/16 or Mn^{2+}, $\alpha5\beta1$-mediated MV3 adhesion to the mutated polypeptide was equally strong as adhesion to the control polypeptide. Mn^{2+} or TS2/16 induced weak K562 binding to the mutated polypeptide, and in the presence of a combination of PMA, Mn^{2+}, and TS2/16, $\alpha5\beta1$-mediated K562 adhesion to the mutated and control polypeptide was equally strong. Our findings demonstrate that requirement for the PHSRN synergy site for $\alpha5\beta1$-mediated adhesion to RGD in fibronectin depends on the activation state of the integrin.

Fibronectin (Fn) is an extracellular matrix (ECM) glycoprotein that functions in cell adhesion and migration in wound healing, embryonic development, and malignant transformation [27,37]. The Fn molecule is composed of three types of repeating modules, termed type I, II, and III repeats [45] which are organized into functional domains. Proteolytic cleavage yields several fragments containing domains that promote cell adhesion, including the carboxyterminal HepII domain [35], the alternatively spliced type III connecting segment [25], and the central cell binding domain (CCBD).

The CCBD consists of type III repeats, each containing approximately 90 amino acids [31]. Cells bind to the CCBD via receptors of the integrin family [26]. Integrins are $\alpha\beta$ heterodimeric transmembrane molecules mediating cell-cell adhesion and attachment of cells to the ECM [28]. Integrins that bind the CCBD include $\alpha 3\beta 1$ [19], $\alpha 5\beta 1$ [2,48], $\alpha v\beta 1$ [60], $\alpha v\beta 3$ [13], $\alpha IIb\beta 3$ [22,23], and $\alpha v\beta 6$ [11].

The Arg-Gly-Asp (RGD) sequence in the 10th type III repeat (3Fn10) is the key attachment site for binding of these integrins to the CCBD as demonstrated by inhibition of cell adhesion with synthetic RGD-containing peptides [47,64]. Furthermore, two synergistic regions in the CCBD besides RGD have been identified that are required for cell adhesion through $\alpha IIb\beta 3$ [8] and $\alpha 5\beta 1$ [3,30,38,40]. For $\alpha 5\beta 1$ binding to Fn, the synergy region in 3Fn9 is the most important of these two regions [3], and recently, a short amino acid sequence Pro-His-Ser-Arg-Asn (PHSRN) was identified in this repeat that synergistically enhances the cell adhesion promoting activity of the RGD sequence [4]. This sequence is also present in an 11 amino acid integrin binding site from 3Fn9 that is recognized by $\alpha IIb\beta 3$ [9].

Integrins do not always constitutively bind to their ligands with high affinity. Integrin adhesiveness can be stimulated by phorbol esters and other more physiologically relevant agonists [18,28]. In addition, antibodies have been described to integrin $\beta 1$ [5,32,39,57], $\beta 2$ [50], and $\beta 3$ [42] subunits, that induce a high affinity state of the integrins. Studies with stimulatory $\beta 1$ antibodies on hematopoietic cells have demonstrated modulation of binding to natural ligands [5,32,57], modulation of ligand specificity [12], modulation of binding to different regions in one ligand [52], and modulation of the minimal sequence of a binding site required for adhesion [62].

In the present study, we have investigated the role of the PHSRN synergy site in $\alpha 5\beta 1$- and $\alpha v\beta 3$-mediated cell adhesion to the CCBD in Fn. We show that requirement for the PHSRN synergy site for cell adhesion to the CCBD depends on the integrins expressed and on the activity of the integrins involved.

Fibronectin, fragments, and peptides

Plasma Fn was purchased from Sigma (St Louis, MO). A polymeric Arg-Gly-Asp (RGD) peptide and a 120 kDa chymotryptic Fn fragment containing the CCBD [46,51], were purchased from Life Technologies (Gaithersburg, MD). For adhesion assays, synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was obtained from the Department of Organic Chemistry, Faculty of Science, University of Nijmegen, The Netherlands, and covalently bound to bovine serum albumin (BSA) as previously described [44]. For adhesion inhibition assays, GRGDSP and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were obtained from Life Technologies.

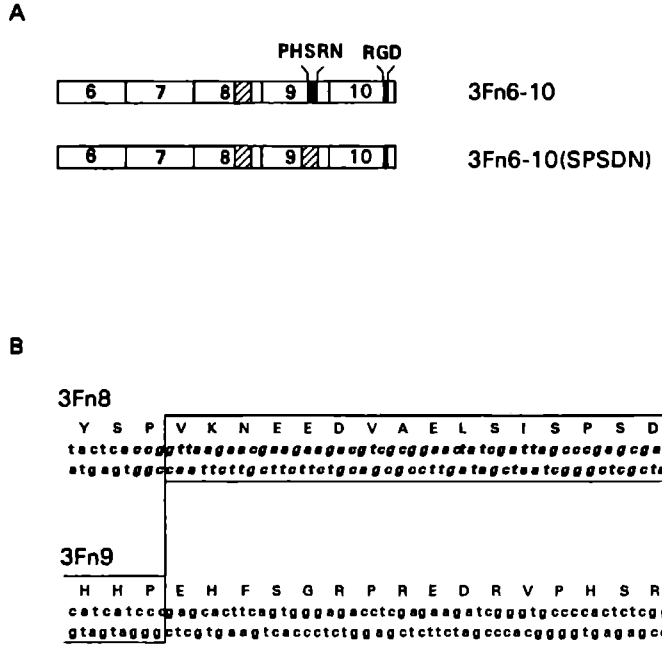


Figure 1. *Recombinant FN polypeptides. A: Schematic representation of a recombinant Fn polypeptide 3Fn6-10 consisting of 5 type III Fn repeats from 3Fn6 through 3Fn10, and of a mutated Fn polypeptide 3Fn6-10(SPSDN) where the region containing PHSRN in 3Fn9 has been substituted by the corresponding region from 3Fn8 (hatched bar). B: In 3Fn6-10(SPSDN), 16 amino acid residues from 3Fn9 were substituted with the corresponding residues from 3Fn8 to disrupt the PHSRN site in 3Fn9. Boxed sequences are from 3Fn6-10(SPSDN). The region with italicized nucleotides differs from the original sequence in 3Fn8 for technical reasons to alter restriction sites but this does not alter the amino acid sequence.*

Production of recombinant fibronectin polypeptides

In order to avoid the artifactual losses of adhesive activity known to result from adsorbing short polypeptides on substrates (e.g. see [38]), we used recombinant Fn polypeptides containing five type III Fn repeats from 3Fn6 through 3Fn10. The 3Fn6-10 wildtype expression construct was generated based on the T7 phage promotor and a Fn cDNA fragment encoding Fn type III repeat numbers 6-10 produced using the polymerase chain reaction method [4]; the PHSRN sequence was present in repeat 9 and RGD in repeat 10 (Fig 1a). The N-terminal sequence of 3Fn6 starts immediately after an initiation codon for methionine. To create substitution mutants, two complementary oligonucleotides with appropriate sequences were synthesized, annealed, and then cloned between the BamH1 and EcoR1 sites of 3Fn9. This yielded a mutated polypeptide 3Fn6-10(SPSDN) where the PHSRN sequence from 3Fn9 was substituted by SPSDN from 3Fn8 (Fig 1a,b).

Protein expression was induced by 1 mM ITPG treatment of E. coli strain BL21 (DE3, pLysS) containing the expression plasmid. The expressed recombinant polypeptides were purified by sequential DEAE and hydroxyapatite column chromatography. The polypeptide was eluted from a DEAE column (DE52, Whatman) using a linear gradient of 0 to 0.5 M NaCl in 10 mM sodium phosphate (pH 7.4), 1 mM EDTA, 0.02% sodium azide, then applied to a hydroxyapatite column (Bio-Rad) and eluted using a linear gradient from 5 mM sodium phosphate (pH6.5), 0.4 mM EDTA, 0.02% sodium azide to 250 mM sodium phosphate (pH 6.5), 0.4 mM EDTA, 0.02% sodium azide. The fractions with peak absorbance were evaluated for purity by SDS-PAGE, pooled, dialysed against PBS without Ca^{2+} or Mg^{2+} and with 0.02% sodium azide, and stored at $-80°C$.

Cell lines and culture conditions

The human melanoma cell lines used included Mel57 [10] and MV3 [59]. The K562 erythroleukemic cell line was provided by Dr. Nancy Hogg. All cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) (Flow, Irvine, UK) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Antibodies

Anti-integrin antibodies included P1B5 anti-α3 [61], purchased from Telios Pharmaceuticals Inc. (San Diego, CA); HP2/1 anti-α4 [53], provided by Dr. Francisco Sanchez-Madrid; NKI-Sam1 anti-α5 [57], provided by Dr. Carl Figdor; 4B4 anti-β1 [36], purchased from Coulter Immunology (Hialeah, FL); AJ2 anti-β1 [29], provided by Dr. Eberhard Klein; C17 anti-β3 [56], provided by Dr. Arnoud Sonnenberg; A109 polyclonal anti-αv [54], purchased from Life Technologies; 10E5 anti-αIIb [15], provided by Dr. Barry Coller; and LM142 anti-αv and LM609 anti-$\alpha v\beta$3 [14], provided by Dr. David Cheresh. The stimulatory anti-integrin β1 mAbs were 8A2 [32], provided by Dr. Nicholas Kovach; and TS2/16 [24], provided by Dr. Francisco Sanchez-Madrid. Anti-Fn

mAbs were 16G3 and 13G12 [38].

Cell adhesion

Cell adhesion assays were performed as described previously [16]. In short, polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with the appropriate adhesive ligands and blocked for 1 h at 37°C with DMEM containing 0.5% wt/vol BSA. Subsequently, $1x10^4$ ^{51}Cr-labeled cells in 50 μl DMEM/BSA were added to the wells and incubated for 30 min at 37°C in 5% CO_2. Unbound cells were removed by washing with DMEM/BSA, bound cells were lysed by detergent, and radioactivity of the lysate was measured in a gamma counter. Results are presented as the mean percentage of cell binding from triplicate wells. For induction of adhesion, radiolabeled cells were either preincubated with TS2/16 or 8A2 mAbs for 30 min at 4°C before seeding in the wells, or 1 mM $MnCl_2$, or 100 ng/ml phorbol 12-myristate 13-acetate (PMA) was added to the cells prior to seeding in the wells. For adhesion inhibition studies, cells were preincubated with the appropriate mAbs or peptides for 30 min at 4°C before seeding into the wells.

Flow cytometry

Cells were incubated with mAbs in PBS containing 0.5% wt/vol BSA and 0.02% wt/vol sodium azide for 30 min at 4°C. After washing with PBS/BSA/azide, the cells were incubated with fluorescein-isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) for 30 min at 4°C. After washing, fluorescence was measured on an Epics Elite flow cytometer (Coulter, Mijdrecht, The Netherlands).

Transfection

The full-length cDNA for the integrin $\beta 3$ subunit [58], a kind gift from Dr. Erkki Ruoslahti, was cloned in the polylinker of the mammalian expression vector pBJ1neo [33], kindly provided by Dr. René de Waal-Malefijt. Twenty μg of this construct was used for stable transfection of MV3 cells according to the calcium phosphate precipitation method [63], using the Calcium Phosphate Transfection System (Life Technologies). After 48h, stably transfected cells were selected by culturing in the presence of 1 mg/ml G418 (Life Technologies) for 2 weeks. Cell populations were enriched for $\alpha v\beta 3$ expression by cell sorting in an Epics Elite flow cytometer using LM609 mAbs. After three cycles of sorting, transfected cell lines contained >95% $\alpha v\beta 3$ positive cells. Cells were maintained in culture in medium containing 200 μg/ml G418 and regularly monitored for $\alpha v\beta 3$ expression.

K562, MV3, and Mel57 differentially adhere to the CCBD

We investigated adhesion of K562 human erythroleukemic cells and MV3 and Mel57 human melanoma cells to the CCBD. Of the integrins known to be involved in adhesion to Fn, K562 exclusively expressed $\alpha5\beta1$ (Table 1). MV3 and Mel57 expressed $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$. In addition, Mel57 but not MV3 expressed $\alpha v\beta3$. MV3 and Mel57 expressed other αv integrins including $\alpha v\beta5$ [17] and possibly $\alpha v\beta1$ that may bind to Fn.

Table 1. *Fn-binding integrins on K562, MV3 and Mel57 cells.*

	mean fluorescence							
	control	α3	α4	α5	αv	αIIb	β1	αvβ3
K562	4	4	4	46	7	3	52	3
MV3	4	69	31	62	13	3	107	2
Mel57	3	41	17	23	33	3	33	41

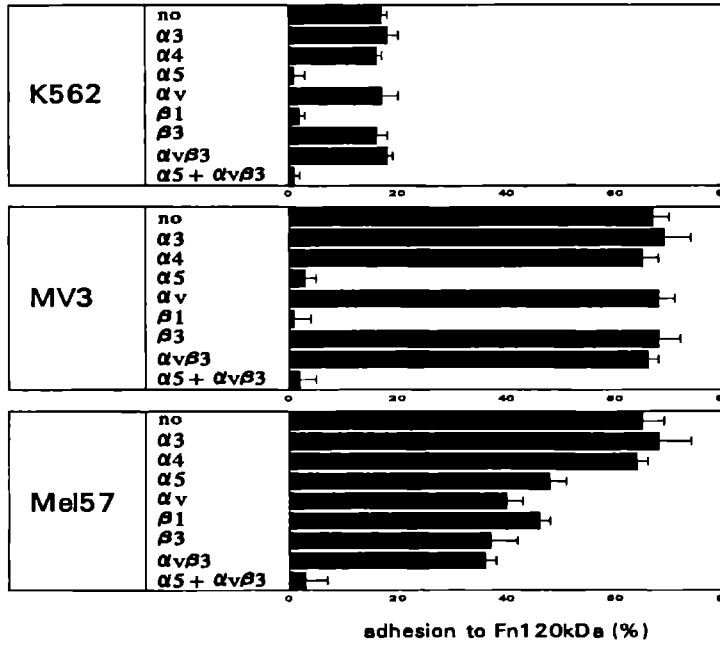


Figure 2. *Inhibition of adhesion to Fn120kDa with integrin mAbs. Cells were allowed to adhere to wells coated with 20 μg/ml of a 120 kDa fragment of Fn (Fn120kDa) in the absence (no) or in the presence of inhibitory mAbs to integrin subunits as indicated. Adhesion to BSA was less than 5%. One experiment of 4 is shown.*

In order to exclude influences from domains outside the CCBD that are known to have cell adhesive activity (HepII, IIICS), we used a 120 kDa Fn fragment that lacks the heparin-binding domain and the V-region but includes the CCBD. K562 adhered weakly to Fn120kDa, whereas MV3 and Mel57 both adhered strongly (Fig 2). As expected from the surface expression data, adhesion of K562 was completely blocked by mAbs to $\alpha 5$ or $\beta 1$. Even though MV3 expressed several Fn-binding integrins, adhesion was fully blocked by mAbs to $\alpha 5$ whereas mAbs to $\alpha 3$ or $\alpha 4$ or polyclonal anti-αv had no effect. Adhesion of Mel57 was inhibited approximately 35% by mAbs to $\alpha 5$ or $\beta 1$, and about 50% by mAbs to $\beta 3$ or $\alpha v \beta 3$ or by polyclonal anti-αv. The combination of mAbs to $\alpha 5$ and $\alpha v \beta 3$ completely blocked adhesion of Mel57.

Thus, K562 adheres weakly to the CCBD through $\alpha 5 \beta 1$, MV3 binds strongly through $\alpha 5 \beta 1$, and Mel57 binds strongly through $\alpha 5 \beta 1$ and $\alpha v \beta 3$.

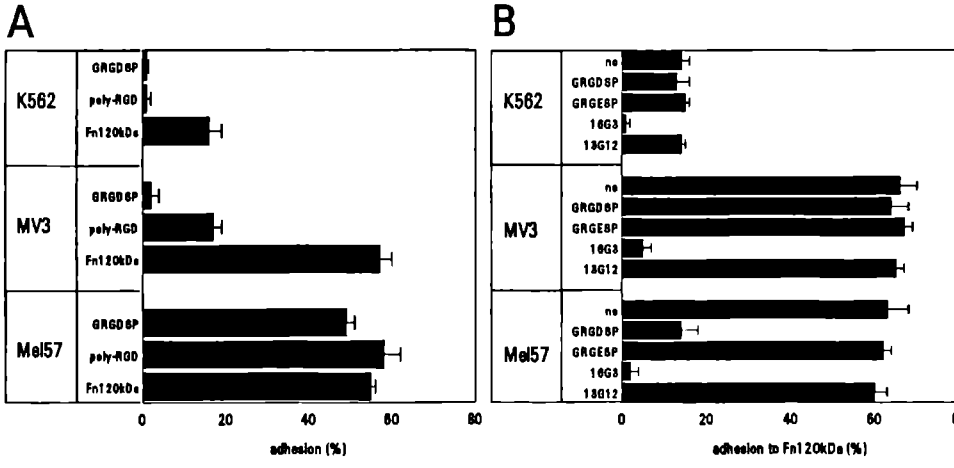


Figure 3. Adhesion to RGD-containing ligands. A: Cells were allowed to adhere to 20 μg/ml of GRGDSP peptide coupled to BSA, to polymeric RGD, or to Fn120kDa. Adhesion to BSA was less than 4%. One representative experiment of three is shown. B: Cells were incubated in the absence (no) or presence of 500 μg/ml GRGDSP, a control peptide GRGESP, or 16G3 or 13G12 anti-Fn mAbs as indicated, and allowed to adhere to wells coated with 20 μg/ml Fn120kDa. Adhesion to BSA was less than 4%. One experiment of 3 is shown.

K562 and MV3 require the PHSRN synergy site whereas for Mel57 RGD is sufficient

To study binding of these cells to the cell recognition site RGD, we used a GRGDSP peptide and polymeric RGD as adhesive ligands. K562 did not adhere to either of the

ligands and MV3 bound only weakly to polymeric RGD (Fig 3a). In contrast, Mel57 adhered strongly to both ligands. When soluble GRGDSP was used to inhibit adhesion to Fn120kDa, it only blocked adhesion of Mel57 whereas adhesion of K562 and MV3 was not influenced (Fig 3b). A control GRGESP peptide had no effect. Inhibition with the 16G3 anti-Fn mAb, which binds in close proximity to the RGD site [38], demonstrated that all cell lines bind to that region in the CCBD (Fig 3B). A control anti-Fn mAb, 13G12, which binds at greater distance from the RGD site [38], had no effect.

To further study the mechanism of binding to the CCBD, we used a recombinant Fn polypeptide containing 3Fn6-3Fn10 and a mutated polypeptide lacking the recently described PHSRN synergy site [4] (Fig 1). As shown in figure 4, K562 did not adhere to the mutated polypeptide and weakly to the control polypeptide. Only very low adhesion of MV3 cells was observed to the mutated polypeptide whereas adhesion of MV3 to the control polypeptide was 5 times higher. In contrast, Mel57 cells adhered strongly to both polypeptides.

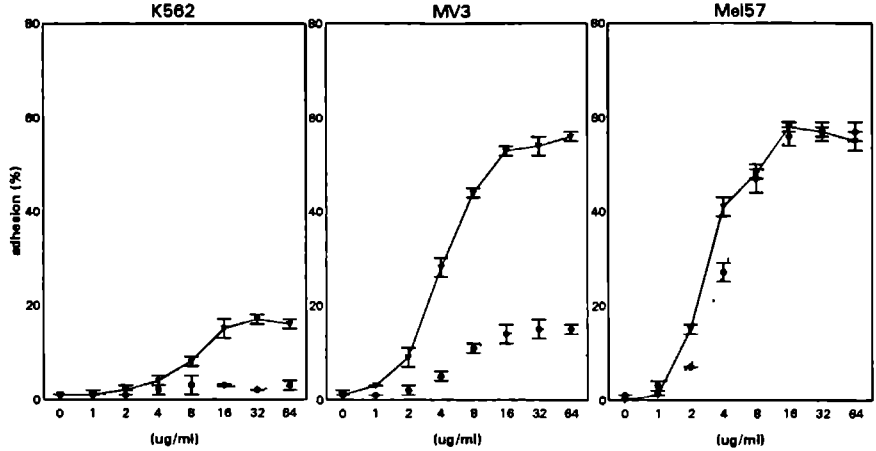


Figure 4. Adhesion to recombinant Fn polypeptides. K562, MV3, or Mel57 cells were allowed to adhere to wells coated with increasing concentrations of 3Fn6-10(SPSDN) (dotted line) or 3Fn6-10 (line) as indicated. Adhesion to 0.1 mg/ml BSA was less than 4%. One experiment of 4 is shown.

To investigate if the different adhesive characteristics of MV3 and Mel57, were due to differential expression of $\alpha v\beta 3$, we transfected MV3 cells with $\beta 3$ cDNA, resulting in $\alpha v\beta 3$ surface expression (Fig 5a), and used these cells in adhesion assays. Expression of

αvβ3 provided MV3 cells with the capacity to adhere to GRGDSP (not shown) and to the mutated polypeptide, and this adhesion could be inhibited by C17 anti-β3 mAbs (Fig 5b). A similar level of inhibition was found with LM609 anti-αvβ3 (not shown).

From these results, we conclude that the differential requirement for the PHSRN synergy site for adhesion to RGD in the CCBD of MV3 versus Mel57, is due to the different binding mechanisms of α5β1 versus αvβ3.

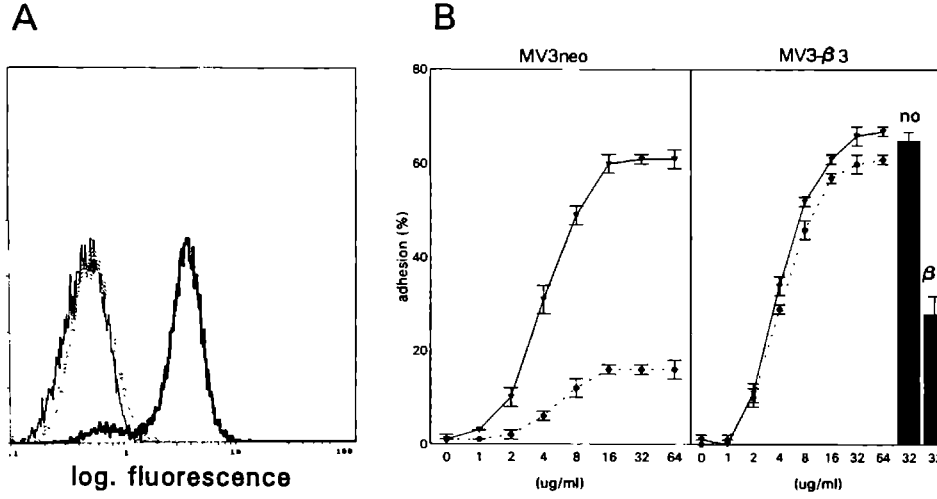


Figure 5. Expression of αvβ3 on MV3 induces adhesion to 3Fn6-10(SPSDN). A: MV3 cells were either untransfected (dotted line), transfected with pBJ1neo alone (thin line), or transfected with pBJ1neo including integrin β3 cDNA followed by sorting with LM609 anti-αvβ3 mAbs (thick line). Shown is the relative fluorescence after incubation with LM609 and a FITC-labeled second antibody. B: MV3neo or MV3-β3 cells were allowed to adhere to wells coated with increasing concentrations of 3Fn6-10(SPSDN) (dotted line) or 3Fn6-10 (line) as indicated. Filled bars represent remaining adhesion to wells coated with 32 μg/ml of 3Fn6-10(SPSDN) in the presence of inhibitory anti-integrin mAbs as indicated. Adhesion to BSA was less than 3%. One experiment of 3 is shown.

Stimulation of α5β1-mediated RGD binding with anti-β1 mAbs, PMA, and manganese

The fact that K562 did not adhere to the mutated polypeptide whereas MV3 did to a low extent (Fig 4), even though both cell lines used α5β1, suggested that binding of α5β1 to RGD in 3Fn10 might depend on the activation state of the integrin. To investigate this, we treated both cell lines with 8A2 and TS2/16 stimulatory β1 mAbs, with Mn^{2+}, or with PMA, prior to using them in adhesion assays to a GRGDSP peptide. PMA had no effect, 8A2 and TS2/16 induced weak adhesion of K562 to GRGDSP, and treatment of MV3 cells with these mAbs resulted in 25% adhesion to GRGDSP (Fig 6a). A control β1-integrin mAb AJ2 had no effect (not shown). The strong binding of Mel57 was not

enhanced by 8A2 or TS2/16. Mn^{2+} was less effective for K562 but induced adhesion of MV3 cells up to 35%. We performed adhesion inhibition assays to examine whether the effect of 8A2 and TS2/16 was due to activation of $\alpha5\beta1$ or to the recruitment of other integrins. Induced adhesion of K562 to GRGDSP in the presence of 8A2 (Fig 6b) or TS2/16 (not shown) was blocked by mAbs to $\alpha5$ and not by any of the other mAbs. In addition, even though 8A2 and TS2/16 may activate $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and possibly $\alpha v\beta1$ on MV3 cells, induced adhesion of MV3 to GRGDSP was completely blocked by mAbs to $\alpha5$ whereas mAbs to $\alpha3$, $\alpha4$, and αv had no effect (Fig 6b). The fact that the 4B4 anti-$\beta1$ mAb did not inhibit adhesion in the presence of 8A2, is in line with the report that activating and inhibiting antibodies share a common epitope on the $\beta1$ subunit [55].

Thus, the strength of $\alpha5\beta1$ binding to RGD can be increased by Mn^{2+} and by activating $\beta1$ antibodies.

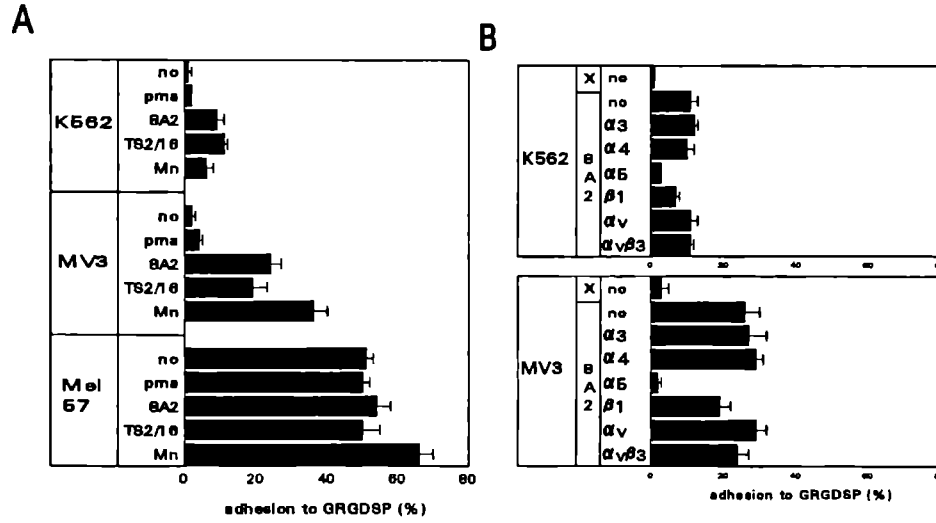


Figure 6. Stimulation of $\alpha5\beta1$-mediated adhesion to RGD. A: Cells were incubated in the absence (no) or in the presence of PMA, 8A2 or TS2/16 stimulatory $\beta1$ mAbs, or manganese (Mn) and allowed to adhere to wells coated with 20 μg/ml BSA-GRGDSP. B: Cells that had been previously incubated in the absence (x) or in the presence of 8A2 stimulatory $\beta1$ mAbs (8A2), were incubated in the absence (no) or in the presence of inhibitory anti-integrin mAbs as indicated and allowed to adhere to wells coated with 20 μg/ml BSA-GRGDSP. Adhesion to BSA was less than 5%. One experiment of 3 is shown.

Requirement for the PHSRN synergy site depends on the activation state of $\alpha5\beta1$

As stimulatory $\beta1$ mAbs and Mn^{2+} induced $\alpha5\beta1$-mediated adhesion to GRGDSP, we hypothesized that the activation state of $\alpha5\beta1$ determines the requirement for the PHSRN synergy site for cell adhesion to the CCBD. Therefore, we treated K562 and MV3 cells with PMA, TS2/16, or Mn^{2+}, and allowed them to adhere to the mutated and control Fn polypeptides. TS2/16 and, to a lesser extent, Mn^{2+}, induced adhesion of K562 cells to the mutated polypeptide and enhanced adhesion to the control polypeptide (Fig 7). PMA enhanced adhesion of K562 cells to the control polypeptide but had no effect on adhesion to the mutated polypeptide. For MV3 cells, no effect of PMA was observed but the low adhesion to the mutated polypeptide was enhanced 5-fold by TS2/16 and Mn^{2+}, resulting in a level of adhesion that was similar to that observed with the fully active control polypeptide.

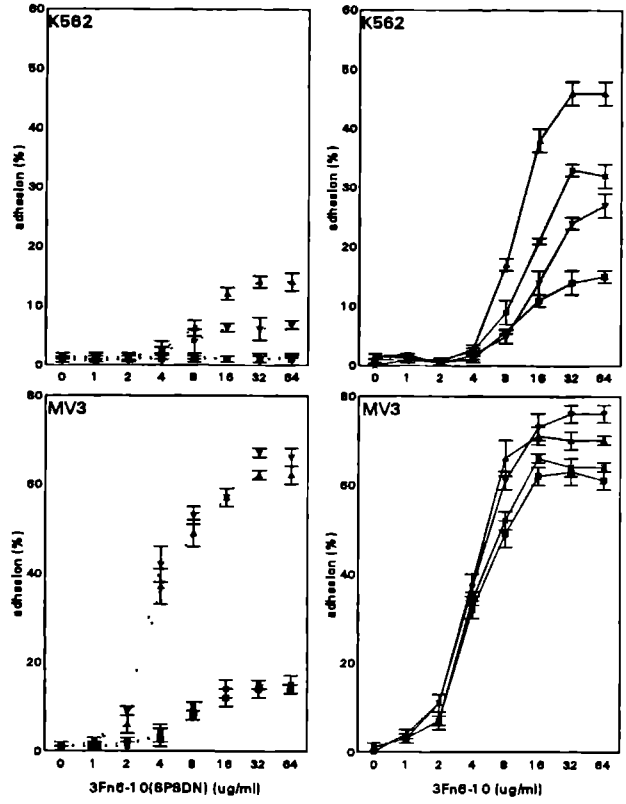


Figure 7. Stimulation of adhesion to recombinant Fn polypeptides. Cells were incubated in the absence (■) or in the presence of PMA (*), TS2/16 (▲), or manganese (▼), and allowed to adhere to wells coated with increasing concentrations of 3Fn6-10(SPSDN) (dotted line) or 3Fn6-10 (line) as indicated. Adhesion to BSA was less then 4%. One experiment of 3 is shown

The fact that in the presence of TS2/16 or Mn^{2+}, no difference was observed between the mutated and control polypeptide regarding adhesion of MV3 cells, whereas for K562 the mutated polypeptide was still poorly active, could suggest a) that stimulation of MV3 cells resulted in recruitment of other RGD-binding integrins, or b) that $\alpha5\beta1$ on K562 cells was not maximally activated by these agents. To exclude possibility a, we used mAbs to $\alpha3$, $\alpha4$, $\alpha5$, αv, $\beta1$, $\beta3$, $\alpha v\beta3$, or the combination of these mAbs in the absence of anti-$\alpha5$, for inhibition of TS2/16-stimulated adhesion of MV3 cells to the mutated polypeptide. Stimulated adhesion was blocked by the anti-$\alpha5$ mAb and not by any of the other mAbs or their combination (Fig 8), suggesting that induction of adhesion to the mutated polypeptide of MV3 by TS2/16 was due to activation of $\alpha5\beta1$ and not to recruitment of other integrins.

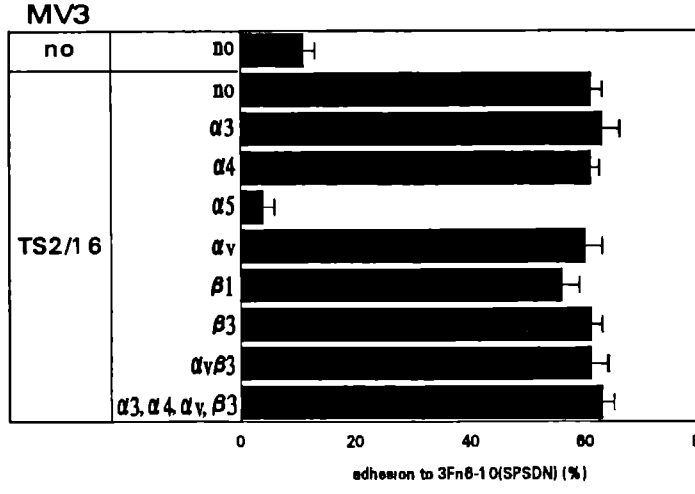


Figure 8. Inhibition of stimulated adhesion of MV3 to 3Fn6-10(SPSDN) with integrin mAbs. MV3 cells were incubated in the absence or in the presence of TS2/16 and allowed to adhere to wells coated with 32 μg/ml 3Fn6-10(SPSDN). Inhibitory mAbs to integrin subunits were added as indicated. Adhesion to BSA was less then 3%. One experiment of 3 is shown.

To investigate possibility b, we incubated K562 cells with PMA, TS2/16, or Mn^{2+}, and the various combinations, and allowed the cells to adhere to the mutated and control polypeptide. In the presence of the combination of TS2/16 and Mn^{2+}, adhesion to the mutated polypeptide was more than half the level of adhesion to the control polypeptide (Fig 9). PMA had no effect by itself on adhesion to the mutated polypeptide but enhanced adhesion to the control polypeptide more than twofold. Finally, in the presence of the

combination of PMA, TS2/16, and Mn^{2+}, the control and the mutated polypeptide were equally effective in promoting adhesion of K562 cells. This adhesion was blocked by $\alpha 5$ mAbs (not shown).

From these results we conclude that requirement of the PHSRN synergy site for $\alpha 5\beta 1$-mediated adhesion to RGD in the CCBD, depends on the activation state of $\alpha 5\beta 1$.

DISCUSSION

In line with earlier reports, we find that $\alpha v\beta 3$ does not require the PHSRN site. We base this conclusion on 3 observations. First, in contrast to K562 and MV3 that lack $\alpha v\beta 3$ expression, Mel57 cells express $\alpha v\beta 3$ and adhere equally well to all molecules tested containing RGD, i.e. GRGDSP, polymeric RGD, the mutated polypeptide lacking the synergy site 3Fn6-10(SPSDN), the control polypeptide 3Fn6-10, and Fn120kDa. Second, soluble GRGDSP blocks adhesion to Fn120kDa of Mel57 but not of K562 or MV3 cells. Third, MV3 cells do not adhere to RGD-containing ligands that lack the PHSRN site, and transfection with $\beta 3$ cDNA resulting in $\alpha v\beta 3$ surface expression leads to binding of these cells to GRGDSP and 3Fn6-10(SPSDN).

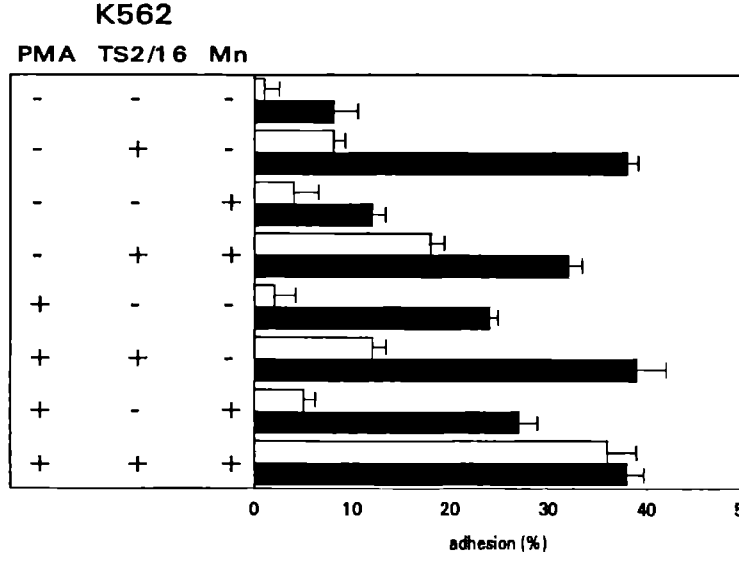


Figure 9. *Stimulation of adhesion of K562 to recombinant Fn polypeptides. K562 cells were incubated in the absence or in the presence of various combinations of PMA, TS2/16, and Mn^{2+} as indicated and allowed to adhere to wells coated with 32 μg/ml 3Fn6-10(SPSDN) (dotted bars) or 3Fn6-10 (filled bars). Adhesion to BSA was less then 4%. One experiment of 3 is shown.*

These findings confirm and extend the observations that $\alpha v\beta 3$ can be retained on an RGD column [49] whereas $\alpha 5\beta 1$ cannot [48]. Furthermore, these data are in agreement with the recent report that αv- and α3- but not α5-containing integrins are bound by a column containing a Fn-fragment lacking the synergy region [1]. Similarly, it has been reported that $\alpha IIb\beta 3$ but not $\alpha v\beta 3$ binding to Fn can be inhibited by an 11 amino acid peptide from 3Fn9 that also contains the PHSRN sequence [9]. Thus, RGD is sufficient for binding to Fn through $\alpha v\beta 3$, whereas $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ require the synergy region for efficient binding to Fn [4,9].

Parenthetically, it has been reported that crosstalk between $\alpha v\beta 3$ and $\alpha 5\beta 1$ can occur [6,7]. Therefore, the induced adhesion to 3Fn6-10(SPSDN) upon expression of $\alpha v\beta 3$ in MV3 cells did not necessarily have to be due to $\alpha v\beta 3$-mediated adhesion. Even though Blystone et al. [7] show that $\alpha v\beta 3$ regulates only $\alpha 5\beta 1$-mediated phagocytosis, in our system $\alpha v\beta 3$ might influence $\alpha 5\beta 1$-mediated adhesion. Ligation of $\alpha v\beta 3$ with LM609 mAbs might induce a signal that inhibits $\alpha 5\beta 1$. To exclude this possibility, we used C17 anti-$\beta 3$ for adhesion inhibition assays. The fact that these mAbs inhibit adhesion of $\beta 3$-transfected MV3 cells to 3Fn6-10(SPSDN) suggests that direct binding through $\alpha v\beta 3$ rather than signalling to $\alpha 5\beta 1$ is involved.

The major conclusion from this study is that the requirement for the PHSRN synergy site for $\alpha 5\beta 1$-mediated adhesion to the CCBD depends on the activation state of $\alpha 5\beta 1$. This is based on 3 findings. First, stimulation of K562 cells that express only $\alpha 5\beta 1$, with Mn^{2+} or stimulatory $\beta 1$-integrin mAbs, induces adhesion to GRGDSP and 3Fn6-10(SPSDN). Second, in the presence of the combination of PMA, TS2/16, and Mn^{2+}, the mutated and control polypeptide are equally effective in promoting K562 cell adhesion. Third, treatment of MV3 cells with these agents induces adhesion to GRGDSP and enhances adhesion to 3Fn6-10(SPSDN) to the level of adhesion to 3Fn6-10, and this effect is completely blocked by antibodies to $\alpha 5$ but not by mAbs to $\alpha 3$, $\alpha 4$, or αv, or the combination.

Even though the $\alpha v\beta 3$-negative K562 and MV3 cells express similar levels of $\alpha 5\beta 1$, they differ dramatically in binding to Fn120kDa through this receptor. The view of cell type specific regulation of $\alpha 5\beta 1$ affinity proposed by O'Toole et al. [43] suggests that the default low affinity state of the integrin as observed in K562 is switched to a high affinity state in MV3. As a result, MV3 but not K562 cells bind strongly to Fn120kDa. Our finding that K562 cells bind poorly to Fn120kDa and that 8A2 increases that adhesion 2-3 times is in line with earlier findings [20]. As expected, Mn^{2+} and stimulatory $\beta 1$ mAbs do not affect the strong adhesion of MV3 to Fn120kDa. However, our findings demonstrate that these agents do in fact alter the avidity of $\alpha 5\beta 1$ in MV3 cells, but that this change can only be observed in the absence of the PHSRN synergy site. One interpretation of these findings is that intracellular factors (induced by PMA for K562 and factors already present in MV3) can increase the affinity of $\alpha 5\beta 1$ to a level where RGD is recognized in the Fn molecule, and that additional extracellular events are required for the final

activation of $\alpha5\beta1$ leading to full adhesion to RGD in Fn. The synergy site could be involved in the last step by locking the RGD site in the $\alpha5\beta1$ binding pocket, and in the presence of TS2/16 or Mn^{2+} that last step seems to be no longer required. Our finding that PMA enhances K562 adhesion to the control polypeptide whereas by itself it has no effect on adhesion to the mutated polypeptide, is in line with this idea. Furthermore, the fact that K562 cells in the presence of TS2/16 bind strongly to the control polypeptide without the need for PMA, demonstrates that optimal extracellular stimulation (the synergy site plus stimulatory $\beta1$ mAbs) can abrogate the need for intracellular activation (PMA).

It is of interest that comparable observations have been reported for $\alpha4\beta1$ [62]. Even though Jurkat and Ramos cells express an active form of $\alpha4\beta1$ in the sense that they are capable of binding to the CS1 domain of Fn, they only bind to a peptide containing the EILDV recognition sequence from CS1 in the presence of stimulatory $\beta1$ mAbs. The authors suggest that sequences may be present in the NH_2-terminal portion of CS1 that strengthen $\alpha4\beta1$ binding to EILDV, although none have yet been identified. Thus, the presence of sites that synergistically enhance binding of integrins to their recognition sequence might be a general mechanism, and activation by stimulatory $\beta1$ mAbs and Mn^{2+} may bypass the dependence on such sites. Our report, however, provides the first example of substitution of the function of a well-characterized synergy site by agents that activate the integrins involved.

For leukocytes, stimulatory $\beta1$ mAbs also increase the affinity of $\alpha4\beta1$ for CS1 [34,62] and for VCAM-1 [32], and they can even induce $\alpha4\beta1$ binding to the RGDS sequence [52]. MV3 cells adhere to CS1 and TNFα-stimulated endothelial cells in the absence of stimuli (not shown) indicating the expression of active $\alpha4\beta1$ on these cells. For MV3 cells in the absence or presence of stimuli, we do not observe any inhibition of binding to the CCBD with HP2/1 anti-$\alpha4$ mAbs, whereas these mAbs inhibit binding to CS1 (not shown). Thus, the reported recognition of RGD by stimulated $\alpha4\beta1$ does not play a role in our assay. This difference may be explained by the fact that Ramos cells, as used by Sanchez-Aparicio et al. [52], do not express $\alpha5\beta1$. In MV3, the effect of TS2/16 on $\alpha4\beta1$ may be masked by the binding to RGD through $\alpha5\beta1$. Alternatively, as reported previously [21], stimulatory $\beta1$ mAbs may selectively activate $\alpha5\beta1$ while leaving $\alpha4\beta1$ unaffected.

A possible interpretation of our findings may be that the PHSRN synergy site binds to the same epitope as recognized by the stimulatory $\beta1$ mAbs. It has been suggested previously that the epitope where these mAbs bind may physically interact with extracellular proteins [55]. However, the fact that binding of K562 to 3Fn6-10 can be enhanced in the presence of 8A2 or TS2/16 when PMA is absent, indicates that the synergy site and stimulatory mAbs can have additional combined stimulatory effects. Therefore these data seem to support a model where the synergy site and stimulatory mAbs have different binding sites on $\alpha5\beta1$. This is in agreement with the recent report

that the mechanism of binding of integrin $\alpha 5\beta 1$ to Fn seems to be through binding of the $\alpha 5$ subunit to the synergistic regions and of the $\beta 1$ subunit to RGD [41].

In conclusion, our data demonstrate that $\alpha 5\beta 1$ but not $\alpha v\beta 3$ requires the PHSRN synergy site for cell adhesion to RGD in the CCBD of Fn, but that induction of a high affinity state of $\alpha 5\beta 1$ with PMA, stimulatory mAbs, and/or Mn^{2+}, abrogates this dependence on the PHSRN sequence.

ACKNOWLEDGMENTS

We thank Drs. David Cheresh, Barry Coller, Carl Figdor, Eberhard Klein, Nicholas Kovach, Francisco Sánchez-Madrid, and Arnoud Sonnenberg for kindly providing antibodies. We thank Dr. René de Waal-Malefijt for the pBJ1-neo expression vector, Dr. Erkki Ruoslahti for the $\beta 3$-cDNA, and Dr. Nancy Hogg for the K562 cell line. We are indebted to Mr. Arie Pennings for expert assistance in the flow cytometric cell sorting procedure and to Dr. Carl Figdor for critical reading of the manuscript. This work was supported by the Dutch Cancer Society (grant number NUKC 91-09).

REFERENCES

1. Akiyama SK, Aota S, Yamada KM. Function and receptor specificity of a minimal 20 kilodalton cell adhesive fragment of fibronectin. Cell Adh Comm 3, 23-25, 1995.

2. Akiyama SK, Yamada KM. The interaction of plasma fibronectin with fibroblastic cells in suspension. J Biol Chem 260, 4492-4500, 1985

3. Aota S, Nagai T, Yamada KM. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. J Biol Chem 266, 15938-15943, 1991.

4. Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell adhesive function. J Biol Chem 269, 24756-24761, 1994.

5. Arroyo AG, Sànchez-Mateos P, Campanero MR, Martín-Padura I, Dejana E, Sànchez-Madrid F. Regulation of the VLA integrin-ligand interactions through the $\beta 1$ subunit. J Cell Biol 117, 659-670, 1992.

6. Bauer JS, Schreiner CL, Giancotti FG, Ruoslahti E, Juliano RL. Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. J Cell Biol 116, 477-487, 1992.

7. Blystone SD, Graham IL, Lindberg FP, Brown EJ. Integrin $\alpha v\beta 3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha 5\beta 1$. J Cell Biol 127, 1129-1137, 1994.

8. Bowditch RD, Halloran CE, Aota S, Obara M, Plow EF, Yamada KM, Ginsberg MH. Integrin $\alpha IIb\beta 3$ (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. J Biol Chem 266, 23323-23328, 1991.

9. Bowditch RD, Hariharan M, Tominna EF, Smith JW, Yamada KM, Getzoff ED, Ginsberg MH Identification of a novel integrin binding site in fibronectin. Differential utilization by $\beta 3$ integrins. J Biol Chem 269, 10856-10863, 1994.

10. Brüggen J, Sorg C, Macher E. Membrane-associated antigens of human malignant melanoma:

Serological typing of cell lines using antisera from non human primates. Cancer Immunol Immunother 5, 53-68, 1978.

11. Busk M, Pytela R, Sheppard D. Characterization of the integrin αvβ6 J Biol Chem 267, 5790-5796, 1992.

12. Chan BMC, Hemler ME. Multiple functional forms of the integrin VLA-2 can be derived from a single α2 cDNA clone: interconversion of forms induced by an anti-β1 antibody. J Cell Biol 120, 537-543, 1993.

13. Charo IF, Nannizzi L, Smith JW, Cheresh DA. The vitronectin receptor alphav beta3 binds fibronectin and acts in concert with alpha5 beta1 in promoting cellular attachment and spreading on fibronectin J Cell Biol 111, 2795-2800, 1990.

14. Cheresh DA, Harper JR. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDa α-subunit. J Biol Chem 262, 1434-1437, 1987.

15. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 72, 325-338, 1983.

16. Danen EHJ, Van Muijen GNP, Van de Wiel-van Kemenade P, Jansen CFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. Int J Cancer 54, 315-321, 1993.

17. Danen EHJ, Jansen CFJ, Van Kraats AA, Cornelissen IMHA, Ruiter DJ, Van Muijen GNP. Alpha-v integrins in human melanoma: Gain of αvβ3 and loss of αvβ5 are related to tumor progression in situ but not to metastatic capacity of cell lines in nude mice Int J Cancer 61, 491-496, 1995.

18. Diamond MS, Springer TA. The dynamic regulation of integrin adhesiveness. Curr Biol 4, 506-517, 1994.

19. Elices MJ, Urry LA, Hemler ME. Receptor functions for the integrin VLA-3· fibronectin, collagen, and laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations. J Cell Biol 112, 169-181, 1991.

20. Faull RJ, Kovach NL, Harlan JM, Ginsberg MH. Affinity modulation of integrin α5β1: regulation of the functional response by soluble fibronectin. J Cell Biol 121, 155-162, 1993.

21. Faull RJ, Kovach NL, Harlan JM, Ginsberg MH. Stimulation of integrin-mediated adhesion of T lymphocytes and monocytes: two mechanisms with divergent biological consequences. J Exp Med 179, 1307-1316, 1994.

22. Gardner JM, Hynes RO. Interaction of fibronectin with its receptor on platelets. Cell 42, 439-448, 1985.

23. Ginsberg MH, Forsyth J, Lightsey A, Gediak J, Plow EF. Reduced surface expression and binding of fibronectin by thrombin-stimulated thrombasthenic platelets J Clin Invest 71, 619-624, 1983.

24. Hemler ME, Sanchez-Madrid F, Flotte TJ, Kremsky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL. Glycoproteins of 210,000 and 130,000 m.w. on activated T cells. cell distribution and antigenic relation to components on resting cells and T cell lines. J Immunol 132, 3011-3018, 1984

25. Humphries MJ, Komoriya A, Akiyama SK, Olden K, Yamada KM. Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type specific adhesion. J Biol Chem 262, 6886-6892, 1987.

26. Hynes RO. Integrins: a family of cell surface receptors. Cell 48, 549-554, 1987.

27. Hynes RO. Fibronectins, Springer Verlag, New York, 1991.

28. Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. Cell 69, 11-25, 1992.

29. Kantor RRS, Mattes MJ, Lloyd KO. Biochemical analysis of two cell surface glycoprotein complexes, very common antigen 1 and very common antigen 2. Relationship to very late activation T cell antigens. J Biol Chem 262, 15158-15165, 1987.

30. Kimizuka F, Ohdate Y, Kawase Y, Shimojo T, Tagushi Y, Hashino K, Goto S, Hashi H, Kato I, Sekiguchi K, Titani K. Role of type III homology repeats in cell adhesive function within the cell binding domain of fibronectin. J Biol Chem 266, 3045-3051, 1991.

31. Kornblihtt AR, Umezawa K, Vibe-Pedersen K, Baralle FE. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. EMBO J 4, 1755-1759, 1985.

32. Kovach NL, Carlos TM, Yee E, Harlan JM. A monoclonal antibody to $\beta 1$ integrin (CD29) stimulates VLA-dependent adherence of leucocytes to human umbilical vein endothelial cells and matrix components. J Cell Biol 116, 499-509, 1992.

33. Lin AY, Devaux B, Green A, Sagerström C, Elliott JF, Davis MM. Expression of T cell antigen receptor heterodimers in a lipid-linked form Science 249, 677-679, 1990

34. Matsumoto A, Hemler ME. Multiple activation states of VLA-4. J Biol Chem 268, 228-234, 1993.

35. McCarthy JB, Chelberg MK, Mikelson DJ, Furcht LT. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion and heparin binding activities. Biochem 27, 1380-1388, 1988.

36. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.

37. Mosher DA. Fibronectin. Academic Press, San Diego, 1989.

38. Nagai T, Yamakawa N, Aota S, Yamada SS, Akiyama SK, Olden K, Yamada KM. Monoclonal antibody characterization of two distant sites required for function of the central cell binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. J Cell Biol 114, 1295-1305, 1991.

39. Neugebauer KM, Reichardt LF. Cell surface regulation of beta 1-integrin activity on developing retinal neurons Nature 350, 68-71, 1991.

40. Obara M, Kang MS, Yamada KM. Site-directed mutagenesis of the cell binding domain of fibronectin: separable, synergistic sites mediate adhesive function. Cell 53, 649-657, 1988

41. Obara M, Yishizato K. Possible involvement of the interaction of the alpha 5 subunit of alpha 5 beta 1 integrin with the synergistic region of the central cell binding domain of fibronectin in cells to fibronectin binding. Exp Cell Res 216, 273-276, 1995

42. O'Toole TE, Loftus JC, Du X, Glass AA, Ruggeri ZM, Shattil SJ, Plow EF, Ginsberg MH. Affinity modulation of the alpha IIb beta 3 integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor Cell Reg 1, 883-893, 1990.

43. O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ, Ginsberg MH Integrin cytoplasmic domains mediate inside-out signal transduction J Cell Biol 124, 1047-1059, 1994.

44. Peeters JM, Hazendonk TG, Beuvery EC, Tesser GI. Comparison of four bifunctional reagents for coupling peptides to proteins and the effect on the immunogenicity of the conjugtes. J Immunol Methods 120, 133-43, 1989.

45. Petersen TE, Thogersen HC, Skorstengaard K, Vibe-Pedersen K, Sahl P, Sottrup-Jensen L, Magnusson S. Partial primary structure of bovine plasma fibronectin· three types of internal homology Proc Natl Acad Sci USA 80, 137-141, 1983.

46. Pierschbacher MD, Hayman EG, Ruoslahti E. Location of the cell attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell 26, 259-267, 1981.

47. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309, 30-33, 1984

48. Pytela R, Pierschbacher MD, Ruoslahti E Identification and isolation of a 140-kD cell surface glycoprotein with properties expected of a fibronectin receptor. Cell 40, 191-198, 1985.

49. Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science 231, 1559-1562, 1986.

50. Robinson MK, Andrew D, Rosen H, Brown D, Ortlepp S, Stephens P, Butcher EC. Antibody against the leucam B-chain(CD18) promotes both LFA-1- and CR3-dependent adhesion events. J Immunol 148, 1080-1085, 1992.

51. Ruoslahti E, Hayman EG, Engvall E, Cothran WC, Butler WT. Alignment of biologically active domains in the fibronectin molecule. J Biol Chem 256, 7277-7281, 1981.

52. Sanchez-Aparicio P, Dominguez-Jiménez C, Garcia-Pardo A. Activation of the $\alpha 4\beta 1$ integrin through the $\beta 1$ subunit induces recognition of the RGDS sequence in fibronectin. J Cell Biol 126, 271-279, 1994.

53. Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-3· a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. Eur J Immunol 16, 1343-1349, 1986.

54. Suzuki S, Argraves WS, Pytela R, Arai H, Krusius T, Pierschbacher MD, Ruoslahti E. cDNA and amino acid sequences of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors. Proc Natl Acad Sci USA 83, 8614-8618, 1986.

55. Takada Y, Puzon W. Identification of a regulatory region of integrin $\beta 1$ subunit using activating and inhibiting antibodies. J Biol Chem 268, 17597-17601, 1992.

56. Tetteroo PAT, Lansdorp PM, Leeksma OC, Von Dem Borne AEG. Monoclonal antibodies against human platelet glycoprotein IIIa. Br J Haematol 55, 509-521, 1983.

57. Van de Wiel van Kemenade E, van Kooyk Y, de Boer AJ, Huijbens RJF, Weder P, van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J Cell Biol 117, 461-470, 1992.

58. Van Kuppevelt THMSM, Languino LR, Gailit JO, Susuki S, Ruoslahti E. An alternative cytoplasmic domain of the integrin $\beta 3$ subunit. Proc Natl Acad Sci USA 86, 5415-5418, 1989.

59. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. Int J Cancer 48, 85-91, 1991.

60. Vogel BE, Tarone G, Giancotti FG, Gailit J, Ruoslahti E. A novel fibronectin receptor with an unexpected subunit composition ($\alpha v\beta 1$). J Biol Chem 265, 5934-5937, 1990.

61. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. J Cell Biol 105, 1873-1884, 1987.

62. Wayner EA, Kovach NL. Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin. J Cell Biol 116, 489-497, 1992.

63. Wigler M, Silverstein S, Lee LS, Pellicer A, Cheng Y, Axel R. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223-232, 1977.

64. Yamada KM, Kennedy DW. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. J Cell Biol 99, 29-36, 1984.

Inhibition of metastasis of an αvβ3-negative human melanoma cell line by expression of αvβ3 and by the disintegrin eristostatin

Inhibition of metastasis of an αvβ3-negative human melanoma cell line by expression of αvβ3 and by the disintegrin eristostatin

Erik HJ Danen[1], Annemieke A van Kraats[1], Ine MHA Cornelissen[1], Cezary Marcinkiewicz[2], Stefan Niewiarowski[2], Jonathan Pachter[3], Dirk J Ruiter[1], and Goos NP Van Muijen[1]

[1]Department of Pathology, University Hospital, Nijmegen, The Netherlands, [2]Department of Physiology and Sol Sherry Trombosis Center, Temple University Medical School, Philadelphia PA, and [3]Department of Molecular Pharmacology, Schering-Plough Research Institute, Kenilworth, NJ

RGD-containing ligands can inhibit melanoma metastasis though the integrins affected are unknown. For human melanoma, α5β1 and αvβ3 are candidates. They emerge in melanocytic tumor progression and have been implicated in melanoma growth and invasion. We studied the role of these integrins in an experimental metastasis assay using a highly metastatic human melanoma cell line MV3 that lacks αvβ3 but expresses α5β1. As differential expression of these 2 integrins has been reported to modulate in vitro melanoma cell invasiveness, we transfected MV3 with β3-cDNA resulting in αvβ3 surface expression and adhesion to fibrinogen via this receptor. MV3-β3 and two of its clones with high αvβ3 expression were no longer metastatic upon i.v. injection in nude mice. Thus, in line with an earlier report on melanoma cell invasion, αvβ3 can inhibit metastasis of certain human melanoma cell lines. To investigate if α5β1 was critical for MV3 metastasis, we next tested the effect of an anti-α5β1 mAb and peptides specifically binding to this integrin. All reagents blocked adhesion to fibronectin but none of them affected metastasis. Thus, α5β1-mediated adhesion to fibronectin does not seem to be critical for metastasis of αvβ3-negative MV3 melanoma cells. In contrast, the disintegrin eristostatin efficiently blocked MV3 lung metastasis. Eristostatin bound to MV3 cells in vitro and RGD-dependent MV3 adhesion to eristostatin was partially inhibited by anti-α4 mAbs. Eristostatin did not affect α5β1-mediated MV3 adhesion to the central cell binding domain of fibronectin, α4β1-mediated adhesion to the CS-1 domain of fibronectin or to VCAM-1 on stimulated endothelial cells, α2β1-mediated adhesion to collagen, or α6β1-mediated adhesion to laminin. Thus, eristostatin probably does not act by inhibiting the initial attachment of MV3 to endothelial cells or to the subendothelial matrix.

INTRODUCTION

Cell adhesion events are critical in tumor metastasis [55] and integrins play an important role in adhesion of cells to other cells and to the extracellular matrix (ECM) [26]. Many integrins recognize the RGD sequence in their ligands, and it has been shown that synthetic RGD peptides, non-peptide RGD mimetics, or RGD peptides isolated from snake venom called disintegrins, can inhibit experimental metastasis of B16 murine melanoma cells [3,21,24,33,46,54]. $\alpha 2\beta 1$ [7], $\alpha 3\beta 1$ [17], $\alpha 4\beta 1$ [47], $\alpha 5\beta 1$ [1,42], $\alpha v\beta 1$ [63], $\alpha v\beta 3$ [43], $\alpha v\beta 5$ [10], $\alpha v\beta 6$ [6], and $\alpha v\beta 8$ [36] recognize RGD but it is not known which of these integrins is affected. In addition, in contrast to human melanoma cells, B16 cells can express $\alpha IIb\beta 3$ [8,38] an integrin that also binds RGD and has been shown to play a role in metastasis of these cells [23].

In one study, a disintegrin was shown to block experimental metastasis of human melanoma cells [57]. Integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$, are interesting candidates in human melanoma since: a) they are strongly upregulated with melanocytic tumor progression in situ [2,16,49,50], b) antibodies to $\alpha 4$ can inhibit human melanoma cell metastasis [19,37], and c) $\alpha 5\beta 1$ and $\alpha v\beta 3$ have been implicated in growth [18,34] and invasion [51,52] of human melanoma cells. Conversely, expression of $\alpha 4\beta 1$ has been reported to inhibit the early stage of murine melanoma cell metastasis [44] and downmodulation of expression of $\alpha v\beta 3$ has been demonstrated to enhance the invasive capacity of certain human melanoma cells [52].

In the present study we investigated the role of $\alpha v\beta 3$ and $\alpha 5\beta 1$ in human melanoma cell metastasis by transfection and inhibition assays. As the disintegrin eristostatin, that does not bind to $\alpha 5\beta 1$, blocked metastasis of an $\alpha v\beta 3$-negative melanoma cell line, we set out to investigate its mode of action in in vitro assays.

MATERIALS AND METHODS

Proteins, peptides, and antibodies

Plasma fibronectin (Fn) was purchased from Sigma (St Louis, MO). A 120 kDa chymotryptic Fn fragment containing the central cell binding domain and synthetic peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were purchased from Life Technologies (Gaithersburg, MD). A Fn CS-1 peptide coupled to IgG was provided by Dr. Martin Humphries (Manchester, U.K.). Laminin (Ln), isolated from Englebreth-Holm-Swarm mouse sarcoma cells was purchased from Life Technologies, and collagen (Co) type I, isolated from rat tail, was provided by Dr. Eberhard Klein (Würzburg, Germany). Fibrinogen (Fg) type 1 and type 3 were purchased from Sigma and mixed before use. Isolation of disintegrins from crude viper venoms and their affinities for various RGD-binding integrins have been described [41]. Echistatin

binds $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha IIb\beta 3$ with the same high affinity, while bitistatin moderately affects only $\alpha v\beta 3$ and $\alpha IIb\beta 3$, and eristostatin is a very potent inhibitor of $\alpha IIb\beta 3$. The cyclic peptide cRRETAWA that binds specifically to $\alpha 5\beta 1$ [28] was generated as described previously [40].

Anti-integrin mAbs included 5E8 anti-$\alpha 2$ [68], provided by Dr. Richard Bankert (Buffalo, NY); P1B5 anti-$\alpha 3$ [64], purchased from Life Technologies; HP2/1 anti-$\alpha 4$ [48], provided by Dr. Francisco Sanchez-Madrid (Madrid, Spain); NKI-Sam1 anti-$\alpha 5$ [58], provided by Dr. Carl Figdor (Nijmegen, The Netherlands); GoH3 anti-$\alpha 6$ [53], provided by Dr. Arnoud Sonnenberg (Amsterdam, The Netherlands); 4B4 anti-$\beta 1$ [32], purchased from Coulter Immunology (Hialeah, FL); TS2/16 anti-$\beta 1$ [22], provided by Dr. Francisco Sanchez-Madrid; LM142 anti-αv and LM609 anti-$\alpha v\beta 3$ [9], provided by Dr. David Cheresh (La Jolla, CA); P1F6 anti-$\alpha v\beta 5$ [65] purchased from Life Technologies; SN1 anti-$\alpha v\beta 8$ [36], provided by Dr. Steven Nishimura (San Francisco, CA); and 10E5 anti-αIIb [12], provided by Dr. Barry Coller (Stoneybrook, NY). A109 polyclonal anti-αv [56] was purchased from Life Technologies.

E1/6 anti-VCAM-1 and H18/7 anti-E-selectin [45] were provided by Dr. Michael Bevilacqua [La Jolla, CA] and G250 [39], an isotype matched (IgG2b) control mAb for NKI-Sam1, was provided by Dr. Egbert Oosterwijk (Nijmegen, The Netherlands).

Nude mice and cell culture

BALB/c athymic nude mice (nu/nu) were purchased from The Laboratory Breeding and Research Center, G1, Bomholtgaard, Ry, Denmark and kept in separate rooms in cages covered with air filters under specific pathogen-free conditions. Mice were used when 6-8 weeks old. Within a single experiment mice were sex and age matched. The highly metastatic human melanoma cell line MV3 [61] was cultured in Dulbecco's modified Eagles medium (DMEM; Flow, Irvine, UK), supplemented with 10% fetal calf serum (Life Technologies) and antibiotics. Human umbilical vein endothelial cells (HUVEC) were provided by Dr. Hans Westphal, Nijmegen, The Netherlands.

Flowcytometry

Cells were incubated with mAbs in PBS containing 0.5% wt/vol BSA and 0.02% wt/vol sodium azide for 30 min at 4°C. After washing with PBS/BSA/azide, the cells were incubated with fluorescein-isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) for 30 min at 4°C. After washing, fluorescence was measured on an Epics Elite flow cytometer (Coulter, Mijdrecht, The Netherlands). For the disintegrin binding assay, eristostatin was labeled with FITC as described [31]. FITC-eristostatin, separated by gel filtration, fully retained its platelet aggregation inhibitory activity.

Transfection

The full-length cDNA for the integrin $\beta 3$ subunit [59], provided by Dr. Erkki Ruoslahti (La Jolla, CA), was cloned in the polylinker of the mammalian expression vector pBJ1neo [29], provided by Dr. René de Waal-Malefijt (La Jolla, CA). Twenty μg of this construct was used for stable transfection of MV3 cells according to the calcium phosphate precipitation method [66], using the Calcium Phosphate Transfection System (Life Technologies). After 48h, stably transfected cells were selected by culturing in the presence of 1 mg/ml G418 (Life Technologies) for 2 weeks. Bulk- or single cell sorting was done on an Epics Elite flow cytometer using LM609 mAbs. Cells were cultured in medium containing 200 μg/ml G418 and regularly monitored for $\alpha v\beta 3$ expression.

Experimental metastasis assay

To determine the capacity of cells to metastasize, $2x10^6$ tumor cells in 200 μl 0.9% NaCl were injected intravenously (i.v.) into the lateral tail vein. For inhibition assays, cells were mixed with mAbs or peptides 15 min before injection. Mice were killed after 1 month, lungs were formalin fixed and embedded in paraffin, and HE-stained 4 μm sections of 3 levels of the lungs were microscopically examined for lung metastases. In our experience, no other organs are colonized [60,61].

Cell adhesion assay

Cell adhesion assays were performed as described previously [14]. In short, polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with the appropriate adhesive ligands and blocked for 1h at 37°C with DMEM containing 0.5% wt/vol BSA. For adhesion to HUVEC, wells were precoated with 2 μg/ml gelatin for 1h at 37°C followed by $5x10^3$ HUVEC that were allowed to grow for 2-3 days and were incubated for 24h with 10 nM rTNFα (kind gift from Boehringer, Ingelheim, Germany) to induce VCAM-1 expression. Subsequently, $1x10^4$ ^{51}Cr-labeled MV3 cells in 50 μl DMEM/BSA were added to the wells and incubated for 30 min at 37°C in 5% CO_2. Unbound cells were removed by washing with DMEM/BSA, bound cells were lysed by detergent, and radioactivity of the lysate was measured in a gamma counter. Results are presented as the mean percentage of cell binding from triplicate wells. For adhesion inhibition studies, radiolabeled cells were preincubated with the appropriate mAbs or peptides for 30 min at 4°C before seeding into the wells, or HUVEC were preincubated with mAbs for 30 min at 37°C. For induction of adhesion, radiolabeled cells were preincubated with TS2/16 mAbs that induce a high affinity state of $\beta 1$-integrins [55] for 30 min at 4°C before seeding in the wells.

Statistical analysis

Fisher's Exact Test (two-sided) was used for comparison of percentages of mice that developed metastases.

Expression of αvβ3 in MV3 cells inhibits metastasis

The highly metastatic human melanoma cell line MV3 expresses several RGD-bin integrins but it lacks αvβ3 (Table 1).

Table 1. *Expression of RGD-binding integrins on MV3.*

subunit	cIg[2]	α2	α3	α4	α5	β1	αv	αvβ3	αvβ5	αvβ8	αIIb
m.f.[1]	4	20	69	31	62	107	13	2	19	4	3

[1]mean fluorescence (arbitrary units), [2]control Ig.

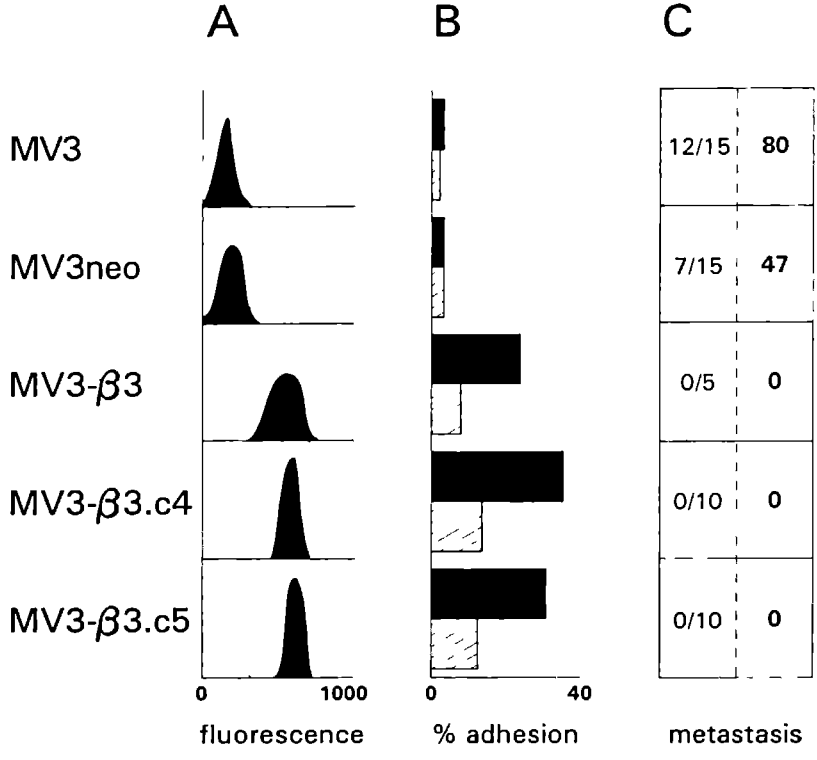


Figure 1. *Expression of αvβ3 in MV3 inhibits experimental metastasis. **A:** MV3 cells were e untransfected or transfected with pBJ1neo alone (MV3neo), or with pBJ1neo including β3-c followed by bulk sorting (MV3-β3) or single cell sorting (MV3-β3.c4 and MV3-β3.c5) with LM anti-αvβ3 mAbs. Shown is the relative fluorescence after incubation with LM609 and FITC-lat second antibody. The dotted vertical line is the gate set with control Ig. **B:** Adhesion of cel wells coated with 100 μg/ml Fg in the absence (filled bars) or presence (hatched bars) of LM anti-αvβ3. **C:** Number and percentage of mice developing lung metastases after i.v. injection.*

In order to investigate the effect of $\alpha v\beta 3$ expression on MV3 metastasis, we transfected $\beta 3$-cDNA into these cells. This resulted in $\alpha v\beta 3$ surface expression (Fig 1a) and it slightly reduced the level of $\alpha v\beta 5$, while no effect was seen on the expression of any of the other integrins (not shown). MV3-$\beta 3$ and 2 clones (MV3-$\beta 3$.c4 and c5), but not the parental or the neo-transfected cell line adhered to Fg, and this adhesion was inhibited by $\alpha v\beta 3$ mAbs (Fig 1b). In vitro proliferation rates of MV3, MV3neo, and MV3-$\beta 3$ were identical; growth of the 2 clones was slightly slower (not shown). In an experimental metastasis assay, 80% of mice injected i.v. with MV3 cells and 47% of mice injected with MV3neo developed lung metastases. In contrast, in the case of MV3-$\beta 3$, MV3-$\beta 3$.c4, or MV3-$\beta 3$.c5, no lung metastasis was observed (Fig 1c).

Thus, expression of $\alpha v\beta 3$ inhibits experimental metastasis of MV3 cells ($p<0.005$ for MV3-$\beta 3$, MV3-$\beta 3$.c4, and MV3-$\beta 3$.c5 compared with parental MV3; $p<0.01$ for MV3-$\beta 3$.c4 and MV3-$\beta 3$.c5 compared with MV3neo).

Table 2. *Inhibition of experimental metastasis of MV3.*

treatment	μg/mouse	exp. 1	exp. 2	exp. 3	total	%	p
no		5/5[2]	4/5	3/5	12/15	80	
eristostatin	25	1/5	0/5	1/5	2/15	13	0.0007
bitistatin	25	5/5	5/5	5/5	15/15	100	>0.05
echistatin	25	4/5	3/5	3/5	10/15	67	>0.05
NKI-Saml	100	5/5	4/5	n.d.	9/10	90	>0.05
cIg[1]	100	4/5	n.d.[3]	n.d.	4/5	80	>0.05
cRRETAWA	1000	3/5	4/5	n.d.	7/10	70	>0.05

[1]control Ig G250, [2]number of mice with lung metastases, [3]not done.

Inhibition of $\alpha 5\beta 1$ does not affect MV3 metastasis

To test the hypothesis that $\alpha 5\beta 1$ may be critical for MV3 metastasis, we injected MV3 cells in the tail vein of nude mice in the presence or absence of reagents that block the adhesive function of $\alpha 5\beta 1$. Incubation for 1h in vitro with each of these reagents, did not affect the viability of the cells. MV3 lung metastasis formation was not affected by $\alpha 5$ mAbs or by the $\alpha 5\beta 1$-specific cyclic peptide cRRETAWA in 2 experiments with 5 mice, or by the $\alpha 5\beta 1$-binding disintegrin echistatin in 3 experiments with 5 mice ($p>0.05$ for all treatments) (Table 2). Amounts of mAbs and disintegrins were similar or larger than those described to be effective in previous studies [3,19,37,54]. All 3 reagents blocked in vitro adhesion of MV3 to Fn and to a 120 kDa Fn fragment containing the central cell binding domain (Fig 2).

These results suggest that blocking $\alpha 5\beta 1$-mediated adhesion to Fn does not inhibit MV3 metastasis.

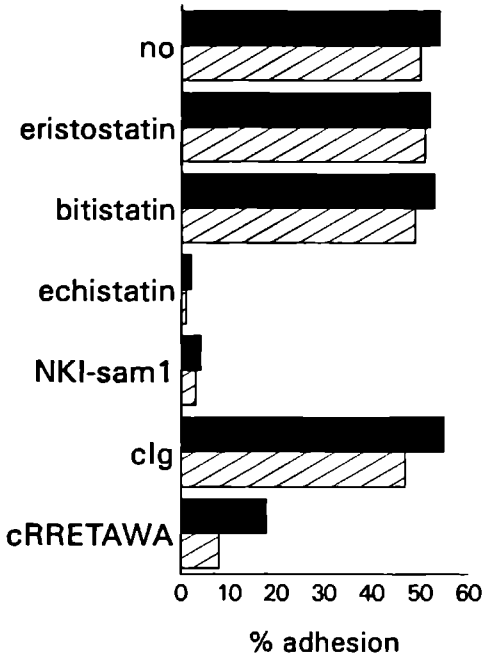


Figure 2. *Inhibition of α5β1-mediated adhesion. MV3 cells were allowed to adhere to wells coated with 20 μg/ml Fn (filled bars) or Fn 120 kDa (hatched bars) in the absence or presence of 10 μg/ml of the disintegrins eristostatin, bitistatin, or echistatin; 5 μg/ml NKI-Sam1 anti-α5 mAb or G250 control Ig (clg); or 100 μg/ml of the cyclic peptide cRRETAWA. S.d. of triplicate determinations did not exceed 5%. One experiment of 3 is shown.*

Eristostatin inhibits MV3 metastasis and binds MV3 cells in vitro

In contrast to these reagents, the disintegrin eristostatin which is known to bind with high affinity to β3 integrins but poorly to α5β1, clearly reduced lung metastasis in all three experiments ($p=0.0007$) (Table 2). A third disintegrin, bitistatin, that also binds β3 integrins but not α5β1, had no effect (Table 2). As MV3 lacks β3 integrins, we tested if these cells could bind eristostatin. As shown in figure 3a, FITC-labeled eristostatin bound to MV3 cells in a concentration-dependent manner. Adhesion of MV3 cells to eristostatin could be inhibited by GRGDSP while a control GRGESP peptide had no effect, but the effect of RGD was low compared to the complete block of adhesion to echistatin and bitistatin (Fig 3b). To identify the RGD-binding integrin on MV3 that was involved in adhesion to eristostatin, we incubated MV3 cells with function-blocking mAbs to various subunits of integrins known to recognize RGD. Some inhibition was observed with HP2/1 anti-α4 mAbs but not with 4B4 anti-β1 (Fig 3c). None of the other mAbs directed to α2, α3, α5, αvβ3, αvβ5, or polyclonal anti-αv had any effect, nor did they enhance inhibition in combination with HP2/1 (not shown).

From these results we conclude that eristostatin binds an RGD-recognizing integrin(s) on MV3, possibly including α4β1, and that it inhibits MV3 experimental metastasis.

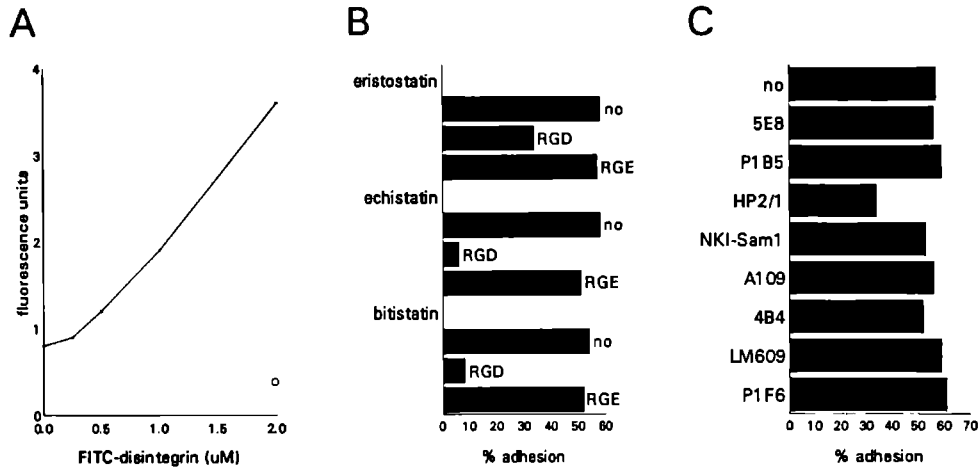


Figure 3. *MV3 binds eristostatin. **A.** MV3 cells were incubated with increasing concentrations of FITC-labeled eristostatin or with FITC-labeled BSA (open circle) as a negative control, and fluorescence was measured. **B.** MV3 cells were allowed to adhere to wells coated with 25 μg/ml of disintegrins as indicated, in the absence or presence of 1 mg/ml GRGDSP or GRGESP. **C.** MV3 cells were allowed to adhere to wells coated with 25 μg/ml eristostatin in the absence or presence of 5E8 anti-α2, P1B5 anti-α3, HP2/1 anti-α4, NKI-Sam1 anti-α5, 4B4 anti-β1, LM609 anti-αvβ3, P1F6 anti-αvβ5 mAbs, or A109 polyclonal anti-αv. S.d. of triplicate determinations did not exceed 5%. One experiment of 2 is shown.*

Eristostatin does not affect in vitro adhesion of MV3 cells

Eristostatin did not affect MV3 adhesion to Fn through α5β1 (Fig 2). As α4β1 in its active conformation has been reported to bind RGD, and as we observed some inhibition of adhesion to eristostatin with HP2/1 anti-α4 mAbs, we next investigated if eristostatin affected α4β1-mediated MV3 adhesive events. Adhesion of MV3 cells to TNFα-stimulated HUVEC that could be blocked with an anti-VCAM-1 mAb and not with a mAb to E-selectin, was not affected by eristostatin (Fig 4a). Similarly, eristostatin did not interfere with adhesion to the CS-1 domain from Fn that was blocked by α4 and β1 mAbs (Fig 4b). As eristostatin-binding to α4β1, and thus its capacity to inhibit α4β1-mediated adhesion, may depend on the activation state of the integrin, we performed the same experiments in the presence of TS2/16 stimulatory β1 mAb. However, incubation of MV3 cells with TS2/16 did not result in eristostatin inhibition of adhesion to HUVEC or CS-1 (not shown).

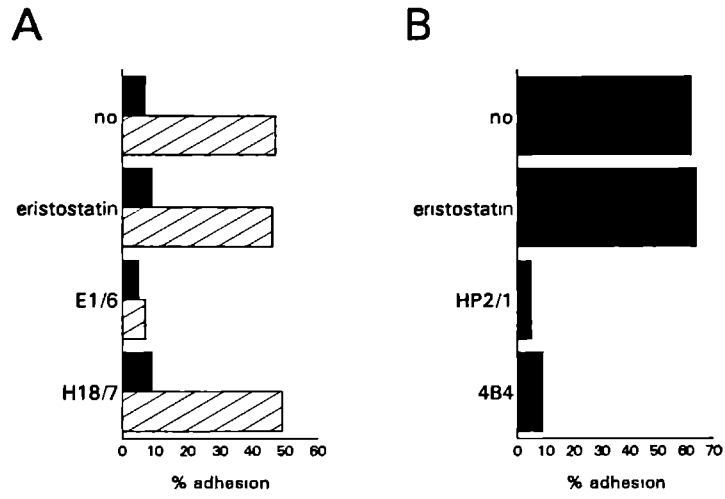


Figure 4. *Inhibition of α4β1-mediated adhesion. A: MV3 cells were allowed to adhere to a monolayer of unstimulated HUVEC (filled bars) or TNFα-stimulated HUVEC (hatched bars) in the absence or presence of 10 μg/ml eristostatin or E1/6 anti-VCAM-1 or H18/7 anti-E-selectin. B: MV3 cells were allowed to adhere to wells coated with 2 μg/ml CS-1-Ig in the absence or presence of 10 μg/ml eristostatin or HP2/1 anti-α4 or 4B4 anti-β1. S.d. of triplicate determinations did not exceed 10% (for A) or 5% (for B). One experiment of 2 is shown.*

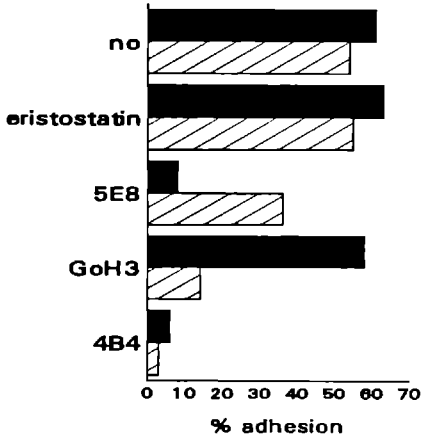


Figure 5. *Inhibition of α2β1-/α6β1-mediated adhesion. MV3 cells were allowed to adhere to wells coated with 20 μg/ml Co (filled bars) or Ln (hatched bars) in the absence or presence of 10 μg/ml eristostatin or 5E8 anti-α2, GoH3 anti-α6, or 4B4 anti-β1. S.d. of triplicate determinations did not exceed 5%. One experiment of 2 is shown.*

In order to investigate if eristostatin affected adhesion of MV3 to basement membrane components, we performed adhesion assays to Co and Ln. Eristostatin did not interfere with α2β1-mediated adhesion to Co (Fig 5). Similarly, α6β1-mediated adhesion to Ln was not affected by eristostatin. Again, incubation with TS2/16 stimulatory β1 mAb did not induce an inhibitory effect of eristostatin (not shown).

From these results we conclude that eristostatin does not affect α2β1, α4β1, α5β1, or α6β1-mediated adhesion of MV3 in vitro.

The fact that MV3 is highly tumorigenic and metastatic [61] and lacks $\alpha v\beta 3$ [15], confirms earlier reports that certain melanoma cell lines can grow and metastasize in the absence of this integrin [5,52]. In contrast, strong expression of $\alpha v\beta 3$ is related to tumorigenicity, invasiveness, and metastatic potential of several other melanoma cell lines [18,20,30,35] and $\alpha v\beta 3$ emerges with melanocytic tumor progression in situ in part of the melanoma cases [2,15,16,49]. Together, these reports demonstrate that $\alpha v\beta 3$ may be important for growth, invasion, and metastasis of some melanoma cell lines, while other melanoma cell lines use alternative mechanisms that do not involve $\alpha v\beta 3$. Moreover, downmodulation of $\alpha v\beta 3$ expression has been shown to enhance A375M melanoma cell invasiveness [52], indicating that $\alpha v\beta 3$ can actually inhibit invasion of some cell lines. Our observation that $\beta 3$-transfected MV3 cells, which express $\alpha v\beta 3$, fail to produce lung metastases, extends this finding and demonstrates that $\alpha v\beta 3$ can inhibit metastasis of certain melanoma cells. A possible explanation for these findings may be that the firm $\alpha v\beta 3$-mediated adhesion to Fn, overrules $\beta 1$-integrin-mediated interactions involved in cell migration on Fn [67]. Alternatively, $\alpha v\beta 3$ signaling may affect the function of other integrins, i.e. $\alpha 5\beta 1$ [4], or it may interfere with the complex of signals derived from other integrins that regulates protease expression and cell proliferation [11,13,27]. For both hypotheses, it is important to realize that in our experiments, $\alpha v\beta 3$ expression is forced onto cells that have adopted a strategy for metastasis in the absence of this integrin. In such a cellular background (integrin/protease profile, etc.) $\alpha v\beta 3$ can be obstructive while it can be part of the metastatic design in other melanoma cells.

Another RGD-recognizing integrin that emerges with melanocytic tumor progression is $\alpha 5\beta 1$. We have recently shown that $\alpha 5\beta 1$ in its active state can bind RGD as strong as $\alpha v\beta 3$ [14], suggesting that it can be a candidate integrin for RGD-inhibition of melanoma metastasis. In benign melanocytic lesions $\alpha 5\beta 1$ is absent while it can be detected in melanomas, with increased expression in advanced stages of tumor progression [2,16,49]. Furthermore, triggering $\alpha 5\beta 1$ can stimulate invasion of $\alpha v\beta 3$-negative C8161 melanoma cells [52]. These findings, and the report that $\alpha 5\beta 1$-binding to Fn stimulates in vitro melanoma cell proliferation [34], suggest that $\alpha 5\beta 1$ may be important for invasion and growth of melanoma cells that lack $\alpha v\beta 3$. It may thus be expected that $\alpha 5\beta 1$ plays a role in MV3 metastasis. However, NKI-Sam1, cRRETAWA, and echistatin do not affect lung metastasis of MV3 cells, while they all bind $\alpha 5\beta 1$ [28,41,58] and inhibit MV3 adhesion to Fn which is exclusively mediated by $\alpha 5\beta 1$ [14]. Thus, our data suggest that binding to Fn through $\alpha 5\beta 1$ is not critical for MV3 metastasis but they do not exclude a possible role for $\alpha 5\beta 1$, as other integrins may take over its Fn-binding function. It has been reported that unoccupied $\alpha 5\beta 1$ inhibits proliferation, while upon Fn-binding, $\alpha 5\beta 1$ generates a growth stimulatory signal [62]. Binding of cRRETAWA and echistatin may mimic Fn-binding but the absence of inhibition with the $\alpha 5$ mAb suggests that neither adhesion nor

proliferative signaling through $\alpha 5\beta 1$ is critical.

Earlier studies have shown that eristostatin inhibits murine melanoma cell metastasis [3,33]. Our study extends these findings and demonstrates that eristostatin can also inhibit metastasis of human melanoma cells in a nude mouse model. It has been reported that eristostatin in a dose of 500 ng per mouse provided almost complete protection against metastases and that it was 80 times more potent than echistatin on a molar basis [3]. In that study, eristostatin appeared to be an extremely potent inhibitor of B16F10-induced murine platelet aggregation, and it had little effect on adhesion to ECM components. Beviglia et al. [3] suggested that eristostatin may prevent metastasis by disrupting melanoma-platelet aggregates, formed by fibrinogen bridging αIIb$\beta 3$ on platelets and other fibrinogen-binding integrins on B16F10 cells. Our current study suggests that the anti-metastatic activity of eristostatin does not involve a platelet-dependent mechanism since MV3 cells lack $\beta 3$ integrins that are implicated in melanoma-platelet interaction [8,23]. Accordingly, Morris et al. [33] excluded that the inhibition of B16F1 liver metastasis observed with eristostatin, involved inhibition of platelet aggregation. Further evidence against a role for inhibition of tumor-platelet interactions in the effect of RGD-containing ligands, is the fact that RGD-peptides have been demonstrated to inhibit B16 metastasis in platelet-depleted mice [25].

There is one previous report on inhibition of human melanoma cell metastasis by a disintegrin [57]. The M24met cells used in that study expressed $\alpha v\beta 3$, but binding of contortrostatin was found to be $\alpha 5\beta 1$-mediated, indicating that blocking $\alpha 5\beta 1$ might have been involved in its in vivo effect. Our findings suggest that such a mechanism is unlikely for eristostatin. We hypothesized that interference with $\alpha 4\beta 1$-mediated adhesion to endothelial cell VCAM-1 might be an alternative mechanism, as it was recently observed that FITC-eristostatin, in contrast to FITC-echistatin, binds $\alpha 4$- (but not mock-) transfected CHO cells [C. Marcinkiewicz and S. Niewiarowski, unpublished observations]. MAbs to $\alpha 4$ have been reported to inhibit TNFα- or IL1-augmented murine melanoma cell metastasis [19,37], and $\alpha 4\beta 1$-expression is associated with poor prognosis in melanoma patients [50], indicating the importance of this integrin for melanoma metastasis. Even though mAbs to $\alpha 4$ partially inhibit MV3 adhesion to eristostatin, $\beta 1$ mAbs have no effect, and MV3 cells do not express $\beta 7$ (not shown). At present we have no explanation for these findings. If $\alpha 4\beta 1$ is involved in binding of eristostatin to MV3, our in vitro adhesion assays suggest that inhibition of $\alpha 4\beta 1$-mediated adhesion to VCAM-1 on endothelial cells or to the Fn CS-1 domain does not play a role in the inhibition of metastasis. As eristostatin does not inhibit in vitro adhesion to Ln and Co either, it does not seem to act by preventing the initial adhesion to vascular endothelial cells or to components of the subendothelial basement membrane. Our findings may support the earlier notion that inhibition of B16F1 liver metastasis by eristostatin is not due to prevention of platelet binding or to interference with extravasation or subsequent invasion of the tumor cells, as was determined by in vivo video-microscopy [33]. Similar to what

was suggested in that study for B16F1 cells, eristostatin might inhibit the outgrowth of individual MV3 cells in the lungs. Studies on the inhibition of MV3 proliferation, migration, and invasion should provide more insight in the mode of action of eristostatin.

In conclusion, our findings demonstrate that a) expression of $\alpha v\beta 3$ can inhibit metastasis of certain human melanoma cells, b) blocking $\alpha 5\beta 1$ on $\alpha v\beta 3$-negative melanoma cells does not result in obstruction of metastasis, and c) the disintegrin eristostatin can block metastasis of human melanoma cells though its mode of action remains to be determined.

ACKNOWLEDGEMENTS

We thank Drs. Richard Bankert, Michael Bevilacqua, Barry Coller, Carl Figdor, Martin Humphries, Eberhard Klein, Steven Nishimura, Egbert Oosterwijk, Francisco Sánchez-Madrid, and Arnoud Sonnenberg for kindly providing antibodies and peptides. We thank Dr. Hans Westphal for HUVEC, Dr. Erkki Ruoslahti for the $\beta 3$ cDNA, and Dr. René de Waal-Malefijt for the pBJ1neo expression vector. We are indebted to Mr. Arie Pennings for expert assistance in the flow cytometric cell sorting procedure. This study was supported by Dutch Cancer Society grant NUKC 91-09 and NIH grant HL45486.

REFERENCES

1. Akiyama SK, Yamada KM. The interaction of plasma fibronectin with fibroblastic cells in suspension. J Biol Chem 260, 4492-4500, 1985.

2. Albelda SM, Mette S, Elder D, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: association of $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

3. Bevilgha L, Stewart GJ, Niewiarowski S. Effect of four disintegrins on the adhesive and metastatic properties of B16F10 melanoma cells in a murine model. Oncol Res 7, 7-20, 1995.

4. Blystone SD, Graham IL, Lindberg FP, Brown EJ. Integrin $\alpha v\beta 3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha 5\beta 1$. J Cell Biol 127, 1129-1137, 1994.

5. Boucherke H, Benchaibi M, Berthier-Vergnes O, Lizard G, Bailly M, McGregor JL. Two human melanoma cell-line variants with enhanced in vivo tumor growth and metastatic capacity do not express the $\beta 3$ integrin subunit. Eur J Biochem 220, 485-491, 1994.

6. Busk M, Pytela R, Sheppard D. Characterization of the integrin $\alpha v\beta 6$ as a fibronectin-binding protein. J Biol Chem 267, 5790-5796, 1992.

7. Cadarelli PM, Yamagata S, Taguchi I, Gosrcan F, Chiang SL, Lobl T. The collagen receptor $\alpha 2\beta 1$ from HG-63 and HT1080 cells interacts with a cyclic RGD peptide. J Biol Chem 267, 23159-23164, 1992.

8. Chang YS, Chen YQ, Timar J, Nelson KK, Grossi IM, Fitzgerald LA, Diglio CA, Honn KV. Increased expression of $\alpha IIb\beta 3$ integrin in subpopulations of murine melanoma cells with high lung-colonizing ability. Int J Cancer 51, 445-451,1992.

9. Cheresh DA, Harper JR. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDa α-subunit. J Biol Chem 262, 1434-1437, 1987.

10. Cheresh DA, Smith JW, Cooper HM, Quaranta V. A novel vitronectin receptor integrin ($\alpha v\beta x$) is responsible for distinct adhesive properties of carcinoma cells. Cell 57, 59-69, 1989.
11. Clark EA, Brugge JS. Integrins and signal transduction: the road taken. Science 268, 233-239, 1995.
12. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 72, 325-338, 1983.
13. Damsky CH, Werb Z. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr Opin Cell Biol 4, 772-781, 1992.
14. Danen EHJ, Aota SH, Van Kraats A, Yamada KM, Ruiter DJ, Van Muijen GNP. Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin $\alpha 5\beta 1$. J Biol Chem 270, 21612-21618, 1995.
15. Danen EHJ, Jansen KFJ, Van Kraats AA, Cornelissen IMHA, Ruiter DJ, van Muijen GNP. αv-Integrins in human melanoma: gain of $\alpha v\beta 3$ and loss of $\alpha v\beta 5$ are related to tumor progression in situ but not to metastatic capacity of cell lines in nude mice. Int J Cancer 61, 491-496, 1995.
16. Danen EHJ, Ten Berge PJM, Van Muijen GNP, Van 't Hof-Grootenboer AE, Bröcker AB, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin-receptor expression in melanocytic tumor progression. Histopathol 24, 249-256, 1994.
17. Elices MJ, Urry LA, Hemler ME. Receptor function for the integrin VLA-3: fibronectin, collagen, and laminin binding are differentially influenced by ARG-GLY-ASP peptide and by divalent cations. J Cell Biol 112, 169-181, 1991.
18. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.
19. Garofalo A, Chirivi RGS, Foglieni C, Pigott R, Mortarini R, Martin-Padura I, Anichini A, Gearing AJ, Sanchez-Madrid F, Dejana E, Giavazzi R. Involvement of the very late antigen 4 integrin on melanoma in interleukin 1-augmented experimental metastasis. Cancer Res 55, 414-419, 1995.
20. Gehlsen KR, Davis GE, Sriramarao P. Integrin expression in human melanoma cells with differing invasive and metastatic properties. Clin Exp Metast 10, 111-120, 1992.
21. Hardan I, Weiss L, Hershkoviz R, Freenspoon N, Alon R, Cahalon L, Reich S, Slavin S, Lider O. Inhibition of metastatic cell colonization in murine lungs and tumor-induced morbidity by non-peptidic arg-gly-asp mimetics. Int J Cancer 55, 1023-1028, 1993.
22. Hemler ME, Sanchez-Madrid F, Flotte TJ, Kremsky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL. Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J Immunol 132, 3011-3018, 1984
23. Honn KV, Chen YQ, Timar J, Onoda JM, Hatfield JS, Fligiel SEG, Steinert BW, Diglio CA, Grossi IM, Nelson KK, Taylor JD. αIIb$\beta 3$ integrin expression and function in subpopulations of murine tumors Exp Cell Res 201, 23-32, 1992.
24. Humphries MJ, Olden K, Yamada KM. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 233, 467-470, 1986.
25. Humphries MJ, Yamada KM, Olden K. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. J Clin Invest 81, 782-790, 1988.
26. Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. Cell 69, 11-25, 1992
27. Juliano RL, Haskill S. Signal transduction from the extracellular matrix. J Cell Biol 120, 577-585, 1993
28. Koivunen E, Wang B, Ruoslahti E. Isolation of a highly specific ligand for the $\alpha 5\beta 1$ integrin from a phage display library. J Cell Biol 124, 373-380, 1993.
29. Lin AY, Devaux B, Green A, Sagerström C, Elliott JF, Davis MM. Expression of T cell antigen receptor heterodimers in a lipid-linked form. Science 249, 677-679, 1990.

30. Marshall JF, Nesbitt SA, Helfrich MH, Horton MA, Polakova K, Hart IA. Integrin expression in human melanoma cell lines. heterogeneity of vitronectin receptor composition and function. Int J Cancer 49, 924-931,1991.

31. McLane MA, Kowalska MA, Silver L, Shattil SJ, Niewiarowski S. Interaction of disintegrins with the αIIbβ3 receptor on resting and activated human platelets. Biochem J 301, 429-436, 1994.

32. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.

33. Morris VL, Schmidt EE, Koop S, MacDonald IC, Grattan M, Khokha R, McLane MA, Niewiarowski S, Chambers AF, Groom AC. Effects of the disintegrin eristostatin on individual steps of hematogenous metastasis. Exp Cell Res 219, 571-578, 1995.

34. Mortarini R, Gismondi A, Santoni A, Parmiani G, Anichini A. Role of the α5β1 integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. Cancer Res 52, 4499-4506, 1992.

35. Nip J, Shibata H, Loskutoff DJ, Cheresh DA, Brodt P. Human melanoma cells derived from lymphatic metastasis use integrin αvβ3 to adhere to lymph node vitronectin. J Clin Invest 90, 1406-1413, 1992.

36. Nishimura SL, Sheppard D, Pytela R. Integrin αvβ8: Interaction with vitronectin and functional divergence of the β8 cytoplasmic domain. J Biol Chem 269, 28708-28715, 1994.

37. Okahara H, Yagita H, Miyake K, Okumura K Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor α enhancement of experimental metastasis. Cancer Res 54, 3233-3236, 1994.

38. Onoda JM, Piechocki MP, Honn KV Radiation-induced increase in expression of the αIIbβ3 integrin in melanoma cells: effects on metastatic potential. Radiat Res 130, 281-288

39. Oosterwijk E, Ruiter DJ, Hoedemaker PJ, Pauwels EKJ, Jonas U, Zwartendijk J, Warnaar SO. Monoclonal antibody G250 recognizes a determinant present in renal cell carcinoma and absent from normal kidney. Int J Cancer 38, 489-494, 1986

40. Pachter JA, Zhang R, Mayer-Ezell R. Scintillation proximity assay to measure binding of soluble fibronectin to antibody-captured α5β1 integrin. Analytical Biochem, in press, 1995.

41. Pfaff M, McLane AM, Beviglia L, Niewiarowski S, Timpl R Comparison of disintegrins with limited variation in the RGD loop in their binding to purified integrins αIIbβ3, αvβ3 and α5β1 and in cell adhesion inhibition. Cell Adhesion Comm 2, 491-501, 1994

42. Pytela R, Pierschbacher MD, Ruoslahti EA. Identification and isolation of a 140 Kd cell surface glycoprotein with properties expected of a fibronectin receptor Cell 40, 191-198, 1985.

43. Pytela R, Pierschbacher MD, Ruoslahti EA. A 125/115 Kda cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. Proc Natl Acad Sci USA 82, 5766-5770, 1985.

44. Qian F, Vaux DL, Weissman I. Expression of the integrin α4β1 on melanoma cells can inhibit the invasive stage of metastasis formation. Cell 77, 335-347, 1994.

45. Rice GE, Bevilacqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246, 1303-1306, 1989.

46. Saiki I, Iida J, Murata J, Ogawa R, Nishi N, Sugimura K, Tokura S, Azuma I. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. Cancer Res 49, 3815-3822, 1989.

47. Sanchez-Aparicio P, Dominguez-Gimenez C, Garcia-Pardo A. Activation of the α4β1 integrin through the β1 subunit induces recognition of the RGDS sequence in fibronectin. J Cell Biol 126, 271-279, 1994.

48. Sanchez-Madrid F, De Landazuri MO, Morago G, Cebrian M, Acevedo A, Bernabeu C. VLA-3: a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. Eur J Immunol 16, 1343-1349, 1986

49. Schadendorf D, Gawlik C, Haney U, Ostmeier H, Suter L, Czarnetzki BM. Tumor progression and

metastasis in vivo correlates with integrin expression on melanocytic tumors J Pathol 170, 429-434, 1993
50. Schadendorf D, Heidel J, Gawlick C, Suter L, Czarnetzki BM Association with clinical outcome of expression of VLA-4 in primary cutaneous malignant melanoma as well as P-selectin on intratumoral vessels. J Natl Cancer Inst 87, 366-371, 1995.
51. Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC Role of the $\alpha v\beta 3$ integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 89, 1557-1561, 1992
52. Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC. The 72 kDa type IV collagenase is modulated via differential expression of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins during human melanoma cell invasion Cancer Res 53, 3411-3415, 1993.
53. Sonnenberg A, Janssen H, Hogervorst F, Calafat J, Hilgers J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. J Biol Chem 264, 13745-13750, 1987.
54. Soszka T, Knudsen KA, Beviglia L, Rossi C, Poggi A, Niewiarowski S. Inhibition of murine melanoma cell-matrix adhesion and experimental metastasis by albolabrin, an RGD-containing peptide isolated from the venom of Trimeresurus albolabris. Exp Cell Res 196, 6-12, 1991.
55. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interaction with the extracellular matrix during invasion and metastasis Annu Rev Cell Biol 9, 541-573, 1993
56. Susuki S, Argraves WS, Pytela R, Arai H, Krusius T, Pierschbacher MD, Ruoslahti E. cDNA and amino acid sequence of the cell adhesion receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors. Proc Natl Acad Sci USA 83, 8614-8618, 1986
57. Trikha M, De Clerck YA, Markland FS. Contortrostatin, a snake venom disintegrin, inhibits $\beta 1$ integrin-mediated human metastatic melanoma cell adhesion and blocks experimental metastasis. Cancer Res 54, 4993-4998, 1994.
58. Van de Wiel van Kemenade E, van Kooyk Y, de Boer AJ, Huijbens RJF, Weder P, van de Kasteele W, Melief CJM, Figdor CG. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA J Cell Biol 117, 461-470, 1992.
59. Van Kuppevelt THMSM, Languino LR, Gailit JO, Susuki S, Ruoslahti E An alternative cytoplasmic domain of the integrin $\beta 3$ subunit Proc Natl Acad Sci USA 86, 5415-5418, 1989.
60. Van Muijen GNP, Cornelissen IMHA, Jansen CFJ, Figdor CG, Johnson JP, Bröcker E-B, Ruiter DJ Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin Expl Metast 9, 259-272, 1991.
61. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. Int J Cancer 48, 85-91, 1991.
62. Varner JA, Emerson DA, Juliano RL. Integrin $\alpha 5\beta 1$ expression negatively regulates cell growth reversal by attachment to fibronectin. Mol Biol Cell 6, 725-740, 1995
63. Vogel BE, Tarone G, Giancotti FG, Gailit J, Ruoslahti E. A novel fibronectin receptor with an unexpected subunit composition ($\alpha v\beta 1$). J Biol Chem 265, 5934-5937, 1990.
64. Wayner EA, Carter WG. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. J Cell Biol 105, 1873-1884, 1987
65. Wayner EA, Orlando RA, Cheresh DA. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface J Cell Biol 113, 919-929, 1991.
66. Wigler M, Silverstein S, Lee LS, Pellicer A, Cheng Y, Axel R. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells Cell 11, 223-232, 1977.
67. Wu CY, Fields AJ, Kapteijn BAE, McDonald JA. The role of $\alpha 4\beta 1$ integrin in cell motility and fibronectin matrix assembly. J Cell Sci 108, 821-829, 1995.
68. Zylstra S, Chen FA, Ghosh SK, Repasky EA, Rao U, Takita H, Bankert RB Membrane associated gp160 identified on human lung tumor by a monoclonal antibody. Cancer Res 48, 2768-2773, 1986

Chapter 10

Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity

Int J Cancer, **61**, 241-248, 1995

Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity

Goos NP van Muijen[1], Erik HJ Danen[1], Jacques H Veerkamp[2], Dirk J Ruiter[1], Jayne Lesley[4], and Lambert PWJ van den Heuvel[2,3]

Departments of [1]Pathology, [2]Biochemistry, and [3]Paediatrics, University Hospital, Nijmegen, the Netherlands, and [4]Department of Cancer Biology, The Salk Institute, San Diego, CA

Changes in glycoconjugate production have been reported for tumor cells. In this study we investigated the glycoconjugate expression pattern in normal human melanocytes and in a panel of 6 human melanoma cell lines with different metastatic capacity after subcutaneous inoculation into nude mice. Glycoconjugates were labeled in vitro with [^{35}S] sulphate and [^{3}H] glucosamine, purified from cells and culture medium by column chromatography and identified by treatment with specific glycosidases. Characterization of the purified glycoconjugate fractions as well as Alcian blue staining of xenograft lesions revealed that hyaluronic acid (HA) is the main glycoconjugate produced by all cell lines. Highly metastatic cell lines expressed higher levels of HA than melanocytes and non or low metastatic cell lines. In addition, a shift from chondroitin sulphate proteoglycan to heparan sulphate proteoglycan dominancy was observed with increasing metastatic capacity. We also studied the expression and binding activity of the HA receptor CD44. Immunoprecipitation experiments indicated a high CD44 synthesis only in highly metastatic cell lines, but flowcytometry demonstrated about the same surface expression in melanocytes and all cell lines. Adhesion assays to immobilized HA showed that CD44 can be present in an inactive or active conformation. Our data suggest that a combination of increased HA production and the expression of CD44 on the cell surface may be associated with a high metastatic potential of human melanoma cell lines in nude mice.

The composition of the cell surface and the microenvironment plays an important role in tumor progression, especially metastasis formation. To create an environment that is conductive to tumor cell invasion, a balance has to be reached between extracellular matrix (ECM) degradation and production of new ECM components. Local disintegration of the ECM network results in expansion and subsequent hydration of the ground substance. In this environment glycosaminoglycans bind water accumulating at the tumor invasion zone due to vascular perturbation [7]. This process is often accompanied and enhanced by the deposition of excessive amounts of hyaluronate (HA) [14,15] and/or chondroitin sulphate (CS) [3,11,19].

In tumors, changes in glycoconjugate expression are prominent at the invasion zone. Not only the production of excessive quantities of various types of normal glycoconjugates by tumor cells has been reported, but also the deposition of chemically altered glycoconjugates, finally resulting in a pathologic ECM [22]. A number of studies have been performed on animal tumor cell lines with different metastatic capacity derived from the same parental line [13,23,24,25,31]. Strongly increased expression of HA was found in highly-metastatic sublines compared with the low-metastatic variants [13,31]. An important and specific role in tumor cell invasion was reported for heparan sulphate proteoglycans (HSPGs) in highly metastatic animal carcinoma and melanoma cell lines [23,24,31], and for chondroitin sulphate proteoglycans (CSPGs) in a highly metastatic lymphoma cell line [25]. Until now only few studies have been published on glycoconjugate expression by human tumor cell lines with different metastatic capacity [3,19,29]. A human melanoma subline with an eight- to tenfold higher capacity to form liver colonies after intrasplenic injection than its parental counterpart, was found to have a dominance of HSPG over CSPG at the cell surface [31].

CD44 can function as a receptor for HA [1,28]. Moreover, it has recently been shown that CD44 and some isoforms of CD44 are associated with tumor cell dissemination in several human malignancies [2,10,33] and in animal tumor model systems [8]. Regarding the involvement of CD44 in melanocytic tumor progression, it has been reported that intravenous (i.v.) inoculation of nude mice with human melanoma cell variants expressing high levels of CD44, gives rise to more extensive lung colonization than with cell line variants expressing low levels of CD44 [2]. However, in a recent study the same group was unable to confirm that association after testing a broad spectrum of different melanoma cell lines [5].

The purpose of this study was to investigate whether a correlation exists between the metastatic potential of human melanoma cell lines and their glycoconjugate expression pattern. Since we found that the level of HA was markedly increased in the frequently metastasizing cell lines, we also studied the expression and functional activity of the HA receptor CD44.

Cell lines and tissue culture conditions

All cell lines were derived from human melanoma metastases and included: IF6; 530; M14; Mel57; BLM and MV3 [4]. All cell lines were cultured in Dulbecco's Modified Eagles Medium (Flow, Irvine, UK) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Isolation and propagation of normal human foreskin melanocytes was performed as described [26] in Ham's F10 (Flow) supplemented with 2% Ultroser-G synthetic serum (GIBCO, Grand Island, NY), antibiotics, 0.1 mM IBMX (Sigma, St. Louis, MO), and 16 nM phorbol 12-myristate 13- acetate (PMA) (Sigma).

[^{35}S] sulphate and [^{3}H] glucosamine labeling of cells

For radioactive labeling of the glycoconjugates, cells were grown to about 80% confluency in 150 cm^2 tissue culture flasks and incubated with 20 ml medium containing 20 μCi/ml [^{35}S] sulphate and 10 μCi/ml [^{3}H] glucosamine (Amersham, Houten, The Netherlands) for 24 h at 37°C. At the end of the labeling culture medium was removed, centrifuged to remove cell debris, and further processed for glycoconjugate analysis. After washing (3x) with phosphate buffered saline (PBS) cells were harvested using a rubber policeman and stored at -80°C until further analysis.

Isolation of glycoconjugates from cells and culture medium

All extractions and subsequent fractionations were performed in the presence of a mixture of protease inhibitors [1 mM phenylmethylsulphonylfluoride (PMSF)/5 mM benzamidine-HCl/10 mM n-ethylmaleimide/0.1 M 6-aminohexanoic acid/5 mM iodo-acetamide/10 mM sodium EDTA].

Labeled human melanoma cells were extracted with 4 M guanidine-HCl/50 mM sodium acetate (pH 5.8) and stirred for 16 h at 4°C. The residue obtained after centrifugation (20,000 x g for 30 min at 4°C) was extracted for an additional 16 h. The combined supernatants were extensively dialyzed at 4°C against 7 M urea/50 mM Tris-HCl (pH 6.8).

Secreted glycoconjugates were isolated from the culture medium. The culture medium was evaporated to dryness and the residue dissolved in 4 ml 7 M urea/50 mM Tris-HCl (pH 6.8). Unincorporated label was removed by gel filtration on a Bio-Gel P2 column (72.0 x 1.6 cm). The peak eluting in the void volume of the column was used for further purification.

Purification of glycoconjugates by Q-Sepharose-HL chromatography

Urea extracts from the melanoma cells or culture medium were chromatographed after centrifugation (100,000 x g for 30 min at 4°C) and filtration through a 0.2 μm filter on a column (1.6 x 10.0 cm) of Q-Sepharose-HL. The following solvents were used for

this FPLC column: A: 7 M urea/50 mM Tris-HCl (pH 6.8) and B: 7 M urea/1 M NaCl/50 mM Tris-HCl (pH 6.8). After application of the sample, a linear gradient was used with a flow rate of 2 ml/min. The gradient started at 0% B for 10 min and rose to 100% B at 60 min. The solvent composition was held at 100% B for 10 min, followed by equilibration back to 0% B. Fractions of 1 ml were collected and glycoconjugates were monitored by determination of the radioactivity in the fractions. The glycoconjugate-containing fractions were pooled, dialyzed against distilled water and stored at -20°C.

Identification of glycoconjugates

Glycoconjugates were identified according to established procedures [32]. Enzymes used were: heparitinase (Seikagaku Kogyo Co, Tokyo, Japan), chondroitinase AC and ABC, and (Streptomyces) hyaluronidase (Sigma). The specificity of the glycosidases was first checked on glycosaminoglycan standards. Heparitinase digestion was performed at 43°C for 18 h in 0.1 M sodium acetate/10 mM calcium acetate (pH 7.0). The enzyme was used at a concentration of 1 U/ml. Chondroitinase ABC digestion was performed at 37°C for 18 h in 0.1 M Tris-HCl (pH 8.0) at an enzyme concentration of 0.25 U/ml. Hyaluronidase digestion was performed at 37°C for 16 h in 50 mM sodium phosphate (pH 5.0); the enzyme was used at a concentration of 10 U/ml. Quantitative proportions of glycosaminoglycans were determined by analyzing the radioactivity after BioGel P100 chromatography.

To test for the presence of glycoconjugates in xenograft lesions, formalin-fixed and paraffin-embedded sections from s.c. tumors and from lungs containing metastases were stained using the Alcian blue technique at pH 2.8. To determine the glycoconjugate profile present in Alcian blue-positive xenograft lesions, sections from s.c. tumors and from lungs containing metastases were preincubated for 1 h (and overnight) with various glycosidases before Alcian blue staining as described above. Parallel sections were treated with HNO_2 for specific chemical degradation of heparan sulphate (HS).

Analytical procedures

Protein content was determined using the Bio-Rad Protein Assay using BSA as a standard.

Antibodies

Control mAbs were WT31 anti-CD3 [26], provided by Dr. Wil Tax (Nijmegen, The Netherlands) for flowcytometry, and 4B4 anti-β1-integrin [20], purchased from Coulter Immunology (Hialeah, FL) for adhesion assays. Anti-CD44 mAbs were NKI-P2 [21], provided by Dr. Carl Figdor (Nijmegen, The Netherlands); 5F12 [18], A1G3 [17], and A3D8 [9], provided by Dr. Barry Haynes (Durham, NC); KM201 [18] from the ATCC; and R7166.7 (Lesley et al., unpublished).

Immunoprecipitation

Subconfluent monolayer cell cultures (75 cm^2) were labeled overnight at 37°C in methionine-free Eagle's minimum essential medium supplemented with 0.3 mCi [^{35}S] methionine and 10% dialyzed FCS. Before harvesting cells were washed twice with PBS. Subsequently NP40-lysis buffer (0.5% NP40, 15 mM NaCl, 10 mM Tris (pH 7.5), 1.0 mM PMSF and 4 μg/ml Aprotinin) was added for 10 min at 4°C resulting in lysis of the labeled cells. Cell debris were scraped out of the culture flask, repeatedly aspirated into syringes and forced through needles with decreasing diameters. Adsorption to Con A-Sepharose (Pharmacia, Uppsala, Sweden) was performed to isolate the glycoprotein fraction from the total cell lysate. Equal numbers of counts of the Con A-bound fraction were used for immunoprecipitation as described earlier [4]. Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel; gels were dried and exposed to X-ray films (Eastman Kodak, Arnhem, The Netherlands) at -70°C using intensifying screens.

Flowcytometry

After short trypsinization of subconfluent monolayers, cells were incubated for 30 min at 4°C with purified mAbs diluted in PBS containing 0.5% BSA and 0.02% sodium azide. After washing with PBS/BSA/azide, cells were incubated with fluorescein-isothyocianate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) and analyzed on an Epics Elite flowcytometer (Coulter Electronics, Mijdrecht, The Netherlands).

Adhesion assay

HA isolated from human umbilical cord was purchased from Sigma. Polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were precoated with 150 μl protamine chloride (200 μg/ml) (KABI AB, Stockholm, Sweden) for 2 h at room temperature. After washing with PBS, 100 μl HA solution (200 μg/ml carbonate buffer, pH 9.6) was added and incubated overnight at room temperature. The plates were then washed with PBS. To avoid non-specific binding, plates were subsequently coated for 2 h at 37°C with 1% (w/v) BSA in PBS and then again washed with PBS. ^{51}Cr-labeled cells [4] were allowed to adhere to the wells for 30 min at 37°C. Non-adhered cells were washed away, attached cells were lysed, and the radioactivity of the lysates was measured in a gamma counter. In adhesion inhibition assays cells were incubated with the appropriate mAbs for 30 min at 4°C before seeding into the wells.

RESULTS

To study the expression of glycoconjugates in relation to metastatic potential, a set of human melanoma cell lines with different metastatic behavior was used. This set included

4 cell lines that are non- or sporadically metastasizing (IF6, 530, M14, Mel57) and 2 cell lines that are frequently metastasizing (BLM, MV3) upon s.c. inoculation into nude mice [4]. In addition, cultured normal human melanocytes were included.

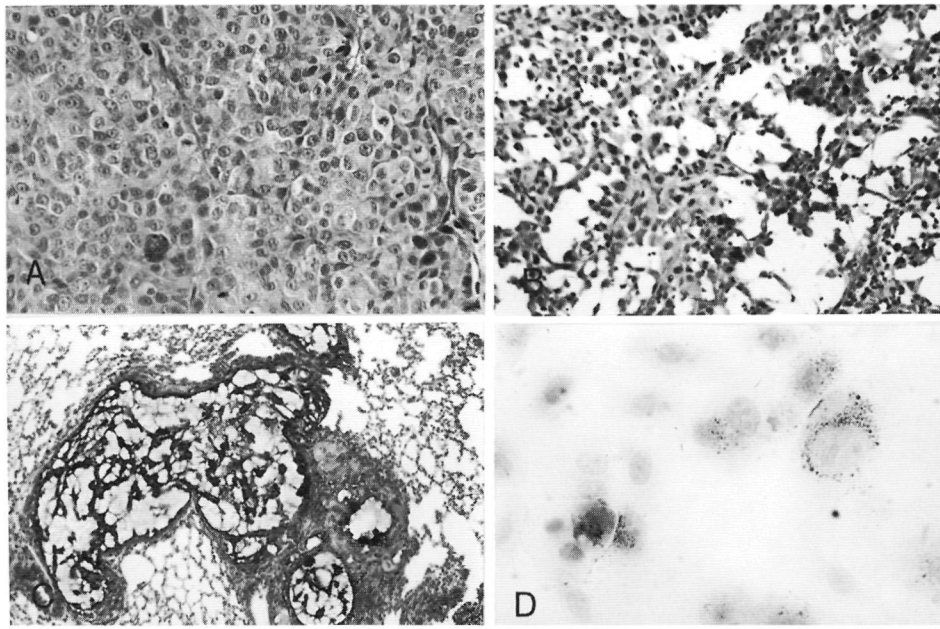


Figure 1. *Histology of BLM xenografts* ***(A,B)*** *and visualization of glycoconjugates in a BLM xenograft* ***(C)*** *and in cultured BLM cells* ***(D)****. HE-stained sections of tumor lesions show some compact areas of tumor cells* ***(A)****, while other parts (of the same lesion) contain dispersed tumor cells with large intercellular spaces* ***(B)****. Alcian blue positivity suggests the presence of glycoconjugates, mainly in the large intercellular spaces* ***(C)****. Bar = 50 μm. Similar results were observed with MV3 cells.*

Expression and identification of glycoconjugates in tumors

Macroscopic examination of s.c. tumors of the 6 melanoma cell lines, demonstrated a marked difference in the consistency of the tumors. While the xenografts of the non- or poorly metastatic cell lines demonstrated a solid aspect, the lesions of the frequently metastatic cell lines showed a mucin-like aspect. Conventionally stained histological sections of the s.c. tumors exhibited exclusively compact areas of tumor cells in IF6, 530, M14 and Mel57 xenograft lesions. In contrast, s.c. tumors and tumor lesions in the lungs of the highly metastatic cell lines BLM and MV3 exhibited besides compact areas (Fig

1a) also parts showing dispersed tumor cells with large intercellular spaces (Fig 1b) containing a mucin-like substance. Alcian blue staining of sections from lesions of all 6 cell lines showed a marked positivity in BLM and MV3 tumors indicating large quantities of glycoconjugates (Fig 1c). No or very weak positivity was seen in tumors derived from the other melanoma cell lines (not shown).

To determine whether the glycoconjugates in BLM and MV3 lesions were produced by the tumor cells or by the tumor stromal cells Alcian blue stainings were performed on cells cultured on coverslips. About half of the tumor cells of both cell lines contained markedly positive intracellular vacuoles (Fig 1d).

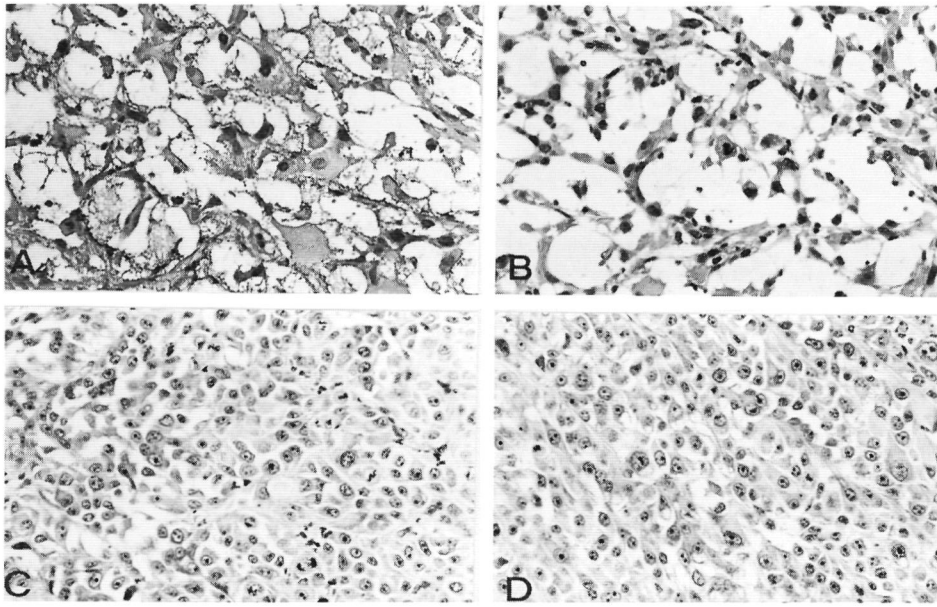


Figure 2. *Alcian blue staining of sections of s.c. lesions of highly metastatic MV3* ***(A,B)*** *and non-metastatic IF6* ***(C,D)*** *cells without* ***(A,C)*** *and after* ***(B,D)*** *treatment with chondroitinase ABC. Note the Alcian blue positivity in the MV3 tumor* ***(A)*** *and the disappearance of positivity after chondroitinase ABC treatment* ***(B)****. Similar results as with chondroitinase ABC were found with chondroitinase AC and hyaluronidase. No disappearance of Alcian blue-positivity was found after pretreatment with heparitinase. No Alcian blue positivity was found in the IF6 tumor* ***(C)****. Bar = 50 μm. Similar results as with MV3 tumors were observed with BLM tumors; 530, M14 and Mel57 tumors showed similar results as IF6 tumors.*

Separate sections were treated with various glycosidases (chondroitinase AC, chondroitinase ABC, hyaluronidase, and heparitinase) to determine the composition of the

glycoconjugates in BLM and MV3 xenograft lesions (Fig 2). Incubation with all enzymes except heparitinase resulted in disappearance of Alcian blue staining. Similar to heparitinase, HNO_2 treatment had no effect. These results indicate that the glycoconjugates in the intercellular spaces of BLM and MV3 lesions represent mainly HA.

Expression and identification of glycoconjugates in vitro

Metabolic labeling with [^{35}S] sulphate and [^{3}H] glucosamine was used to identify the glycoconjugates produced by normal human melanocytes and the different melanoma cell lines in vitro. Purification of the cellular extract on Q-Sepharose-HL resulted in a ^{3}H-peak separated from a ^{35}S-peak for all cell lines (Fig 3). The ^{3}H-peak eluted as a broad peak between 0 and 0.40 M NaCl from the column and the ^{35}S-peak eluted between 0.60 and 0.80 M NaCl. Chromatography of urea extracts from culture media on Q-Sepharose-HL resulted in elution profiles comparable to those observed for the cellular fractions (Fig 3), demonstrating that glycoconjugates were secreted in the culture medium.

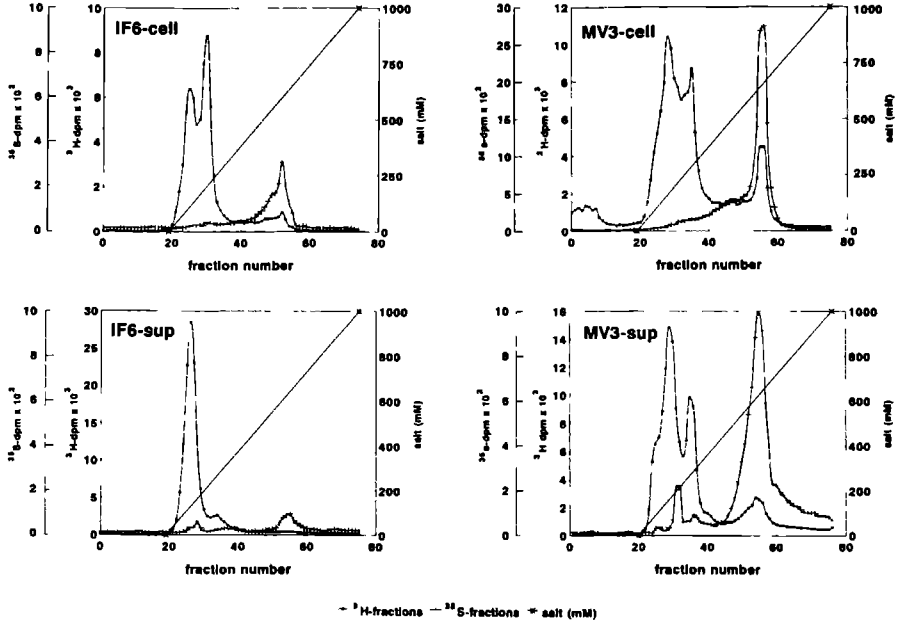


Figure 3. *Chromatography of urea extracts from cells (A,B) and culture media (C,D) on Q-Sepharose-HL after labeling with [^{35}S] sulphate and [^{3}H] glucosamine of IF6 (A,C) and MV3 (B,D) cells. Elution was performed with 7 M urea/50 mM Tris-HCl (pH 6.8) followed by a gradient of 0-1 M NaCl in the same buffer. Fractions (2.0 ml) were analyzed for radioactivity. Fractions from the ^{35}S- and ^{3}H-peak were pooled for further analysis.*

Table 1. Glycosaminoglycan composition of the ^{3}H and ^{35}S peaks.

cell line	cellular fraction		culture medium	
	[^{3}H]HA[1]	ratio [^{35}S]CS/HS[2]	[^{3}H]HA	ratio [^{35}S]CS/HS
melanocytes	259	3.0	162	3.8
IF6	299	2.6	293	3.2
530	365	2.2	291	1.7
M14	301	1.8	438	2.0
Mel57	460	1.3	561	1.4
BLM	623	0.6	726	0.8
MV3	928	0.4	786	0.5

[1]Values are given in dpm/μg protein as means of 3 (IF6, BLM) or 2 (melanocytes, 530, M14, Mel57, MV3) experiments. HA content was determined by hyaluronidase digestion of the ^{3}H-peak.

[2]Ratio CS/HS was determined by digestion of the ^{35}S-peak with chondroitinase ABC and heparitinase. Values are given as means of 2 experiments.

The composition of the ^{35}S-labeled glycoconjugates, from the cellular fraction as well as the culture medium, showed differences between the cell lines studied. A clear change from CS to HS dominancy was observed in the ^{35}S-peak with increasing metastatic capacity of the cell line. This suggests similar changes in the ratio CSPG/HSPG (Table 1). As the ^{3}H-peak, contains other products besides HA originating from [^{3}H] glucosamine, this fraction was treated with hyaluronidase to determine the production of HA. As shown in Table 1, in line with the findings in xenograft lesions, the highly metastatic cell lines showed increased HA production.

Expression of CD44

As the production of HA was elevated in the frequently metastasizing cell lines and as CD44 may function as a receptor for HA, we investigated the presence of CD44. To determine its level of biosynthesis, we performed immunoprecipitation experiments with [^{35}S] methionine-labeled melanocytes and melanoma cells using mAb NKI-P2 which recognizes an epitope present on all CD44 isoforms. As shown in figure 4, a 90 kDa CD44 protein was precipitated from all cell lines, including melanocytes, but synthesis was high in BLM and MV3, intermediate in melanocytes, Mel57 and IF6, and very low in 530 and M14. In addition, we studied surface expression of CD44 using the same mAb in flowcytometry. As shown in figure 5, BLM and MV3 but also the other melanoma cell lines and normal melanocytes had a high level of CD44 surface expression. These data indicate that in addition to the increased synthesis and secretion of HA, synthesis of the CD44 HA-receptor is increased in the highly metastatic cell lines as well, whereas CD44 surface expression is high on all cell lines tested.

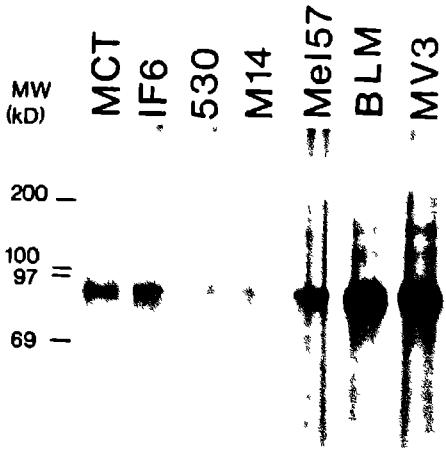


Figure 4. Biosynthesis of CD44 in human melanocytes (MCT) and melanoma cell lines. Subconfluent monolayers were labeled with [^{35}S] methionine. After isolation of glycoproteins from cell lysates with Con A-Sepharose equal numbers of counts were used for immunoprecipitation with mAb NKI-P2.

Adhesion to hyaluronic acid

To investigate whether the expression of CD44 was reflected by the capacity to adhere to HA, we performed adhesion assays to immobilized HA. Two melanoma cell lines, 530 and M14, did not adhere to HA while the other cell lines, including melanocytes, adhered moderately (Mel57) or strongly (melanocytes, IF6, BLM, MV3) (Fig 6). Pretreatment of all cell lines with hyaluronidase or chondroitinase did not alter their binding capacity (data not shown).

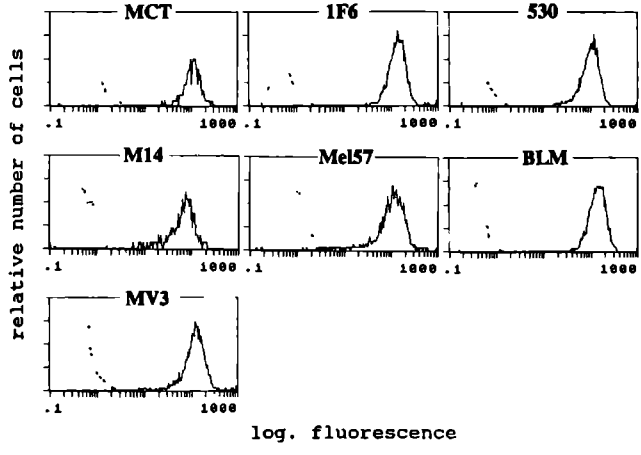


Figure 5. Expression of CD44 on cultured human melanocytes (MCT) and melanoma cell lines. Cells were tested in indirect immunofluorescence with NKI-P2 (anti-CD44) or with WT31 (anti-CD3) as a negative control (dotted line). Following mAb incubation, the cells were exposed to FITC-conjugated rabbit anti-mouse Ig and analyzed by flow cytometry. The fluorescence intensity of 5,000 cells was determined. One representative experiment out of 3 is shown.

To demonstrate that binding to HA was CD44-mediated, adhesion inhibition assays were performed with a panel of 6 different CD44 mAbs. As a negative control 4B4, an adhesion blocking β1 integrin mAb [20] was used. As shown in figure 6, two CD44 mAbs gave partial (5F12) or nearly complete to complete (R7166.7) inhibition of adhesion. No inhibition of adhesion of melanocytes and all melanoma cell lines was found with CD44 mAb KM201 (data not shown) and with the β1 integrin mAb 4B4 although all cells markedly express β1 integrins on their surface [4].

From these results we conclude that even though all cells express CD44 on their surface, CD44-mediated adhesion to HA is highly variable.

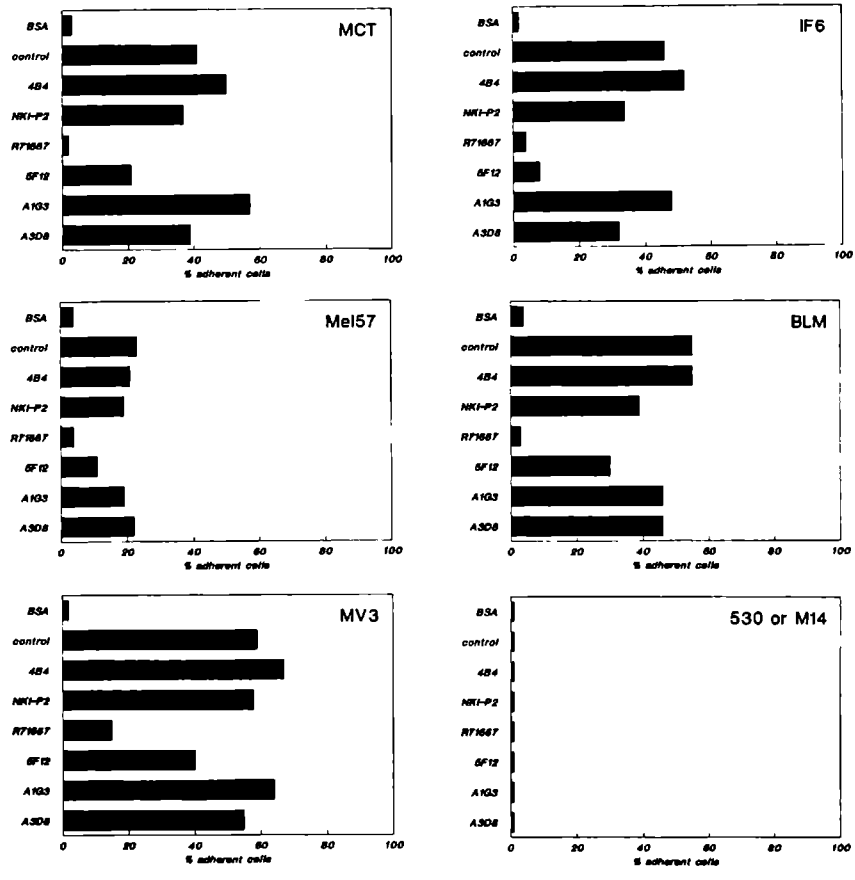


Figure 6. *Adhesion and inhibition of adhesion of human melanocytes (MCT) and melanoma cell lines to HA. BSA=adhesion to wells coated only with BSA. Cells were incubated in the absence (control) or in the presence of mAbs against CD44 (NKI-P2, R7166.7, 5F12, A1G3, A3D8) or β1 integrin (4B4). S.d. of triplicate determinations did not exceed 10%. One experiment of 3 is shown.*

Glycoconjugates are involved in various normal cellular processes, such as cell growth, migration, and adhesion. They may also be involved in various steps of the metastatic cascade. Transformed cells and tumor cells frequently demonstrate altered glycoconjugate patterns, decreased HS and increased HA and CS expression [11]. In addition, both rodent as well as human tumor cell lines with different metastatic capacity express altered glycoconjugate patterns [3,19] especially increased HS/CS ratios with increased metastatic potential [29,30]. Since little is known about glycoconjugate expression by human melanoma cell lines in relation to spontaneous metastasis, in the present study we investigated the expression pattern of melanocytes and a panel of 6 human melanoma cell lines with different metastatic behavior after s.c. inoculation into nude mice [4].

Similar to other studies using low and highly metastatic mouse lymphoma cell lines, Lewis lung tumor cell lines, mouse melanoma cell lines, and various rodent cell lines [23,24,25,31], we find that the production of glycoconjugates is enhanced in highly invasive and metastatic cell lines. In accordance with other studies [30] a shift is observed from CSPG to HSPG dominancy with increasing metastatic capacity.

Enhanced glycoconjugate synthesis has been reported for smooth muscle cells that are cocultured with colon carcinoma cells [12], and for fibroblasts that are cultured in the presence of tumor-conditioned-medium [6]. However, our findings and similar observations by other groups [13,31], indicate that most glycoconjugates in the xenografts are synthesized by the melanoma cells, as only few stromal cells are present in the tumor lesions and as the melanoma cells show a marked production in vitro.

Our finding that the HA receptor, CD44, is expressed on melanocytes and on all melanoma cell lines tested irrespective of their metastatic potential, confirms previous findings [5]. A possible explanation for the higher synthesis in the frequently metastasizing cell lines BLM and MV3, may be that CD44, possibly after binding of "endogenous" HA, is shedded into the culture medium, inducing a continuous synthesis of CD44, but this still has to be proven. It has been reported that CD44 mRNA levels in melanoma cell lines do not correlate with the levels of surface expression [5], suggesting that the control of surface expression is regulated post-transcriptionally. In line with that study, we find that melanoma cells express the standard 90 kD CD44 isoform and that they adhere to immobilized HA through this receptor as proven by inhibition with anti-CD44 mAbs. Regarding the mechanism of inhibition of adhesion by the R7166.7 mAb we know that this is not due to direct blocking of the HA binding site, since the mAb does not block binding of fluorescein-labeled soluble HA to the cell lines (data not shown). Our interpretation of these data is that binding to immobilized HA might be blocked by mAb R7166.7 by restricting CD44 mobility or distribution. Alternatively, steric hindrance may be involved. Our results with the KM201 mAb conflict with the report that this mAb

should inhibit binding of HA to human cells [28]. However, we and others (Kincade; Neame and Isacke, personal communication) were unable to demonstrate KM201 binding to human CD44.

In accordance with East et al. [5] we do not find a correlation between the level of CD44 cell surface expression and the ability of the cells to adhere to HA. Pretreatment of the non or poorly adhering cell lines with hyaluronidase or chondroitinase does not induce or enhance binding to HA, excluding that absence of adhesion is due to binding of "endogenous" HA to the receptor. One explanation for these results is that CD44 is present in an inactive state on the non-adhering cell lines. For murine cells that express CD44 it has been shown that they do not constitutively bind HA, but binding activity can be induced by mAbs recognizing specific epitopes [16]. As activating antibodies to human CD44 were not available to us we could not prove this supposition. Interestingly, East et al. [5] reported a reproducible but not significant increase of the binding capacity of CD44-positive human melanoma cells to HA with 2 different CD44 mAbs.

In conclusion, we found that 1) melanocytes and non- or low metastatic human melanoma cells secrete low levels of glycoconjugates compared with highly metastatic cell lines, 2) there is a shift from CSPG to HSPG dominancy with increasing metastatic capacity, 3) HA is the main glycoconjugate produced by all melanoma cell lines, 4) the synthesis of HA and its receptor, CD44, are markedly higher in the frequently metastasizing cell lines, and 5) CD44 is strongly expressed on all cell lines but seems to be present in an inactive conformation on some cell lines. Our results confirm the potential role of HA and its receptor in the metastatic process.

ACKNOWLEDGEMENTS

We thank Drs. Carl Figdor, Barry Haynes, and Wil Tax for generously providing antibodies. This work was supported by the Dutch Cancer Society (grant NUKC 91-09).

REFERENCES

1. Aruffo AL, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principle cell surface receptor for hyaluronate. Cell 61, 1303-1313, 1990.

2. Birch M, Mitchell S, Hart IR. Isolation and characterization of human melanoma cell variants expressing high and low levels of CD44. Cancer Res 51, 6660-6667, 1991.

3. Caux F, Timar J, Vigny M, Moczar M. Heparan sulfate synthesized by human melanoma cell variants. Cancer J 5, 111-117, 1992.

4. Danen EHJ, Van Muijen GNP, Van de Wiel-Van Kemenade E, Jansen KFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. Int J Cancer 54, 315-321, 1993.

5. East JA, Mitchell SD, Hart IR. Expression and function of the CD44 glycoprotein in melanoma cell lines. Melanoma Res 3, 341-346, 1993.
6. Edward M, Grant AW, Mackie RM. Human melanoma cell-derived factors stimulate fibroblast glycosaminoglycan synthesis. Int J Cancer 52, 499-503, 1992.
7. Gabbert H. Mechanisms of tumor invasion: evidence from in vivo observations. Cancer Metast Rev 4, 293-309, 1985.
8. Günthert U, Hofmann M, Rudy W, Reber S, Zöller M, Hausmann I, Matzku M, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65, 13-24, 1991.
9. Haynes BF, Harden EA, Telen MJ, Hemler ME, Strominger JL, Palker TJ, Scearce RM, Elsenbarth GS. Differentiation of human T lymphocytes. I. Acquisition of a novel human cell surface proteins (p80) during normal intrathymic T cell maturation. J Immunol 131, 1195-1200, 1983.
10. Horst E, Meijer CJLM, Radaszkiewicz T, Ossekoppele GJ, Van Krieken JHJM, Pals ST. Adhesion molecules in the prognosis of diffuse large cell lymphoma: expression of a lymphocyte homing receptor CD44, LFA-1 (CD11a/CD18), and ICAM-1 (CD54). Leukemia 4, 595-599, 1991.
11. Iozzo RV. Proteoglycans and neoplasia. Cancer Metast Rev 7, 39-50, 1988.
12. Iozzo RV, Sampson PM, Schmitt GK. Neoplastic modulation of extracellular matrix: stimulation of chondroitin sulphate proteoglycan and hyaluronic acid synthesis in co-cultures of human colon carcinoma and smooth-muscle cells. J Cell Biochem 39, 355-378, 1989.
13. Kimata K, Honma Y, Okayama M, Oguri K, Hozumi M, Suzuki S. Increased synthesis of hyaluronic acid by mouse mammary carcinoma cell variants with high metastatic potential. Cancer Res 43, 1347-1354, 1983.
14. Knudson W, Biswas CH, Li X-Q, Nemec RE, Toole BP. The role and regulation of tumour-associated hyaluronan. In: D Evered, J Whelan (eds) Biology of hyaluronan, vol 143, pp 150-169. John Wiley and Sons, Chichester, 1989.
15. Konishi I, Fujii S, Nanbu Y, Nonogaki H, Mori T. Mucin leakage into the cervical stroma may increase lymph node metastasis in mucin-producing cervical adenocarcinomas. Cancer 65, 229-237, 1990.
16. Lesley J, Kincade PW, Hyman R. Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody. Eur J Immunol 23, 1902-1909, 1993.
17. Liao H-X, Levesque MC, Patton K, Bergamo B, Jones D, Moody MA, Telen MJ, Haynes BF. Regulation of human CD33H and CD44E isoform binding to hyaluronan by phorbol myristate acetate and anti-CD44 monoclonal and polyclonal antibodies. J Immunol 151, 6490-6499, 1993.
18. Miyake K, Underhill CB, Lesley J, Kinkade PW. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J Exp Med 172, 69-75, 1990.
19. Moczar M, Caux F, Bailly M, Berthier O, Dore JF. Accumulation of heparan sulfate in the culture of human melanoma cells with different metastatic ability. Clin Exp Metastasis 11, 462-471, 1993.
20. Morimoto C, Letvin NL, Boyd AW, Hagan M, Brown HM, Kornacki MM, Schlossman SF. The isolation and characterization of the human helper inducer T cell subset. J Immunol 134, 3762-3769, 1985.
21. Pals ST, Hogervorst F, Keizer GD, Thepen Y, Horst E, Figdor CG. Identification of a widely distributed 90 kD glycoprotein that is homologous to the Hermes-1 human lymphocyte homing receptor. J Immunol 143, 851-857, 1989.
22. Pauli BU, Knudson W. Tumor invasion: a consequence of destructive and compositional matrix alterations. Human Pathol 19, 628-639, 1988.
23. Pogany G, Moczar E, Jeney A, Timar F, Ditroi K, Lapis K. Comparative study on Lewis lung-tumour lines with "low" and "high" metastatic capacity III Glycosaminoglycan synthesis, transport and degradation in cell lines. Clin Exp Metastasis 7, 659-669, 1989.

24. Robertson NP, Starkey JR, Hamner S, Meadows GG. Tumor cell invasion of three-dimensional matrices of defined composition. evidence for a specific role for heparan sulfate in rodent cell lines Cancer Res 7, 1816-1823, 1989.

25. Schwartz-Albiez R, Steffen I, Lison A, Guttler N, Schirrmacher V, Keller R Expression and enhanced secretion of proteochondroitin sulphate in a metastatic variant of a mouse lymphoma cell line Brit J Cancer 57, 569-575, 1988.

26. Smit NPM, Westerhof W, Asghar SS, Pavel S, Siddiqui AH. Large-scale cultivation of human melanocytes using collagen-coated sephadex beads (cytodex 3). J Invest Dermatol 92, 18-21, 1989.

27. Tax WJM, Willems HW, Reekers PPM, Capel PJA, Koene RAP. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 304, 445-447, 1983.

28. Thomas L, Byers HR, Vink J, Stamenkovic I. CD44H regulates tumor cell migration on hyaluronate-coated substrate. J Cell Biol 118, 971-977, 1992.

29. Timar J, Kovalszky I, Paku S, Lapis K, Kopper L. Two human melanoma xenografts with different metastatic capacity and glycosaminoglycan pattern. J Cancer Res Clin Oncol 115, 554-557, 1989.

30. Timar J, Lasanyi A, Lapis K, Moczar M Differential expression of proteoglycans on the surface of human melanoma cells characterized by altered experimental metastatic potential Am J Pathol 141, 467-474, 1992.

31. Turley EA, Tretiak M. Glycosaminoglycan production by murine melanoma variants in vivo and in vitro. Cancer Res 45, 5098-5105, 1985

32. Van den Heuvel LPWJ, Van den Born J, Van de Velden THJAM, Veerkamp JH, Monnens LAH, Schröder CH, Berden JHM. Isolation and partial characterization of heparan sulphate proteoglycan from the human glomerular basement membrane. Biochem J 264, 457-465, 1989.

33. Wielenga VJM, Heider KH, Offerhuis GJA, Adolf GR, Van den Berg FM, Pontah H, Herrlich P, Pals ST. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. Cancer Res 53, 4754-4756, 1993.

Chapter 11

Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential

Int J Cancer, **64**, 182-188, 1995

Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential

Eveliene Manten-Horst[1], Erik HJ Danen[2], Lia Smit[1], Margriet Snoek[3], Carolien Le Poole[1], Goos NP Van Muijen[2], Steven T Pals[1], and Dirk J Ruiter[2]

[1]Department of Pathology, Academic Medical Center, Amsterdam, [2]Department of Pathology, University Hospital, Nijmegen, and [3]Department of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands

Expression of CD44, particularly of certain splice variants, has been linked to tumor progression and metastasis formation in a number of different animal and human cancers. As human cutaneous melanoma is among the most aggressive human cancers, we explored expression of CD44 isoforms (CD44v) in lesions of melanocytic tumor progression. In addition, by RT-PCR and FACS analysis we assessed CD44v RNA species and cell surface expression of CD44v in cultured melanocytes isolated from human foreskin and in a panel of two non-, two sporadically-, and two highly metastatic human melanoma cell lines. We observed that all melanocytic lesions examined, showed strong uniform expression of standard CD44 (CD44s) epitopes. We did not detect CD44v6 expression in the melanocytic lesions. However, CD44 isoforms containing v5 or v10 were differentially expressed. V5 was expressed in 16, 0, 20, 67, and 58% of common nevi, atypical nevi, early primary melanomas ($\leq$1.5mm), advanced primary melanomas (>1.5mm), and metastases respectively and hence was related to tumor progression. In contrast, CD44v10 was expressed in all common nevi whereas part of the atypical nevi and most primary melanomas and metastases lacked v10. CD44v RNA patterns were closely similar in cultured melanocytes and all melanoma cell lines. Melanocytes expressed high levels of CD44s but no CD44v, whereas all melanoma cell lines expressed CD44v at their surface. Interestingly, expression of v5 was strongly increased in the highly metastatic cell lines. Our results may suggest a role for CD44 variant domains, particularly v5 and v10, in human melanocytic tumor progression.

INTRODUCTION

During tumor progression, a subset of cells acquires metastatic properties, presumably through a series of genetic alterations. As a result, cells detach from the primary tumor, penetrate the basement membrane, and invade the connective tissue including lymph- and blood vessels. The tumor cells are subsequently transported to sites of metastatic outgrowth via lymph and/or blood. Loss of existing adhesive functions and gain of new adhesive functions are thought to play a crucial role in this metastatic cascade [12].

CD44, a heterogenous family of molecules with putative functions in cell-cell and cell-matrix interaction has recently been linked to tumor progression in a number of malignancies. In human non-Hodgkin's lymphomas [16,17,29] and in adenocarcinomas of the colon [15,36,42], stomach [23], and breast [18], expression of CD44 was found to be related to tumor dissemination and/or unfavorable prognosis. In a rat model, highly metastasizing adenocarcinoma cell lines express splice variants of the CD44 glycoprotein. These variants differ from the standard CD44 molecule in that they contain additional peptide domains, inserted into the extracellular portion of the transmembrane protein by alternative splicing. Some of these variants play a causal role in the metastatic process. Co-injection of variant-specific mAbs with the metastasizing cells led to retardation or even complete block of metastatic spread in vivo [33]. Moreover, overexpression of specific CD44 variants in non-metastasizing tumor cell lines induced metastatic behavior [11]. CD44 variants, including homologues of those that confer a metastatic phenotype to rat carcinomas, have been found to be overexpressed in human tumors including aggressive non-Hodgkin's lymphomas and colorectal carcinoma [15,22,36,42].

The fact that malignant melanomas are among the most aggressive human tumors, prompted us to explore the expression of CD44 splice variants in human cutaneous melanocytic tumor progression in situ and in cultured normal human melanocytes and melanoma cell lines with different metastatic properties after subcutaneous (s.c.) inoculation into nude mice.

MATERIALS AND METHODS

Antibodies

MAbs directed against the variant portions of CD44 were VFF4 anti-CD44v6 (IgG2b); VFF7 and VFF18 anti-CD44v6 (both IgG1); VFF8 anti-CD44v5 (IgG1); and VFF14 and VFF16 anti-CD44v10 (both IgG1) [22,42]. The mAb against the standard portion of CD44 was NKI-P1 [28]. The specificity of the mAbs is schematically shown in figure 1.

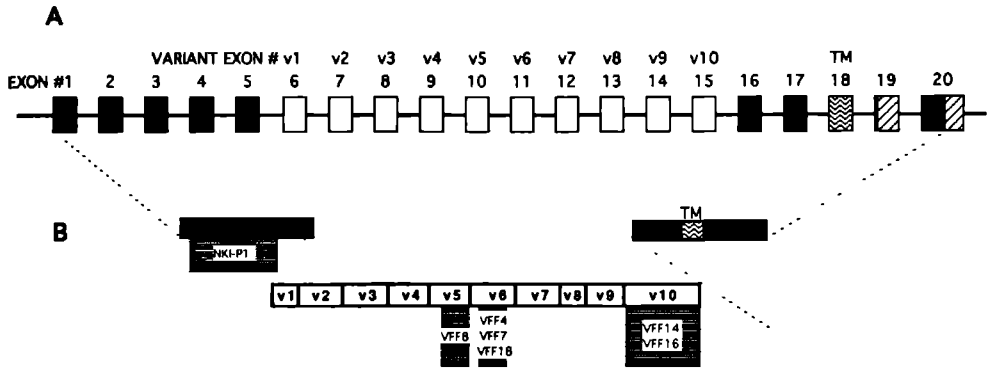


Figure 1. *A: Schematic representation of the CD44 gene. Open boxes indicate exons that are spliced out of the "standard" type of CD44. TM, transmembrane region.* ***B:*** *Schematical representation of the CD44 protein with location of the epitopes which are recognized by the mAb NKI-P1, VFF4, VFF7, VFF8, VFF14, VFF16, and VFF18. Anti-variant antibodies were raised against a bacterially expressed fusion protein encoded by pGEX CD44v HPKII (v3 to v10). Dark area, "standard CD44"; v1-v10, domains encoded by variant exons.*

Tissues

Human melanocytic lesions were selected from the files of the Department of Pathology, University Hospital, Nijmegen, The Netherlands, and the Department of Dermatology, University Hospital, Würzburg, Germany, and tested for expression of CD44s and CD44v, employing the mAbs described above. Based on histopathologic examination of paraffin sections, lesions were divided into five classes: common nevocellular nevus (n=19), atypical (dysplastic) nevocellular nevus (n=9), early primary cutaneous melanoma (i.e. tumor thickness $\leq$ 1.5mm) (n=10), advanced primary cutaneous melanoma (i.e. tumor thickness > 1.5mm) (n=9), and melanoma metastasis (n=19).

Immunohistochemistry

Immunoperoxidase staining was performed as described previously [6]. Briefly, 4 μm cryostat sections were fixed in acetone for 10 min, washed in PBS and incubated with the primary antibody for 1 h. Bound mAbs were visualized using the peroxidase based Vectastain elite ABC Kit (Vector Laboratories, Burlingame, CA) and amino-ethyl-carbazole. Lesions were designated "positive" when they were estimated to show staining in more than 10% of the melanocytic cells.

Cell lines and culture conditions

The human melanoma cell lines 530 and IF6 (non-metastatic) [38,41], M14 and

Mel57 (sporadically metastatic) [5,20] and MV3 and BLM (frequently metastatic) [38,39] were studied for expression of CD44 variants. The cell lines were grown in DMEM (Life technologies, Gaithersburg, MD) supplemented with 1 mM glutamine, 10% (v/v) heat inactivated FCS (Hyclone Laboratories Inc, Logan, UK), and antibiotics. Primary melanocytes were isolated from human foreskin [34] and cultured for a maximum of five passages as described previously [7].

Flowcytometry

Cells were sequentially incubated in PBS containing 1% BSA and 0.02% sodium azide with appropriate dilutions of the various antibodies, followed by incubation with biotin-conjugated rabbit anti-mouse Ig, and phycoerythrin-labelled streptavidin (Dakopatts, Glostrup, Denmark). Fluorescence was measured by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Reverse Transcriptase Polymerase Chain Reaction

5 - 10.10^7 cells were harvested, washed and lysed by the addition of 0.2 ml of RNAzol™ (Campro Scientific, Veenendaal, The Netherlands) per 10^6 cells. Total RNA was extracted by adding 0.2 ml chloroform per 2 ml of cell lysate. After centrifugation at 12,000x g for 15 min (4°C) the aqueous phase was transferred to a fresh tube and RNA was precipitated using isopropanol at -20°C for 45 minutes. Isolated RNA was stored at −80°C in 70% ethanol until use. 2μg aliquots of total RNA were used to synthesize cDNA using an oligo-dT primer with DNA reverse transcriptase, according to the protocol provided with the Perkin Elmer GeneAmp RNA-PCR kit (Perkin Elmer Cetus, CT). Amplification of cDNA was performed using CD44-5' and -3' constant primers corresponding to positions 513-540 and 900-922 of the standard CD44 sequence described by Screaton et al. [30]. Amplification was performed in 35 cycles (30 sec, 94°C; 1 min 55°C; 2 min 72°C) in a microwave-based Ampliwave DNA incubator (Kreatech Biotechnology, Amsterdam, The Netherlands). PCR products were analyzed on a 2% agarose gel (Brunschwig, Amsterdam, The Netherlands) containing ethidiumbromide, subsequently transferred to nylon filters (Gene Screene Plus; NEN Research Products, Boston, MA) and hybridized with a 5'constant CD44-specific oligonucleotide probe or exon v5, v6, v7, and v10 specific oligonucleotide probes labelled with gamma[ATP]^{32}P (Amersham, Den Bosch, The Netherlands) by using polynucleotide kinase (Boehringer, Mannheim, Germany).

RESULTS

Expression of CD44 variants in melanocytic lesions

Immunohistochemical studies demonstrated differential expression of the various

CD44 epitopes in melanocytic lesions (Figs 2,3). In all types of lesions, there was a strong homogenous expression of CD44 proteins as determined by the pan-CD44 mAb NKI-P1 directed against an epitope on the NH_2-terminal constant part of CD44. By contrast, expression of epitopes encoded by v6 was not observed with any of the three mAbs used, although normal epidermal keratinocytes, when present, strongly stained (not shown).

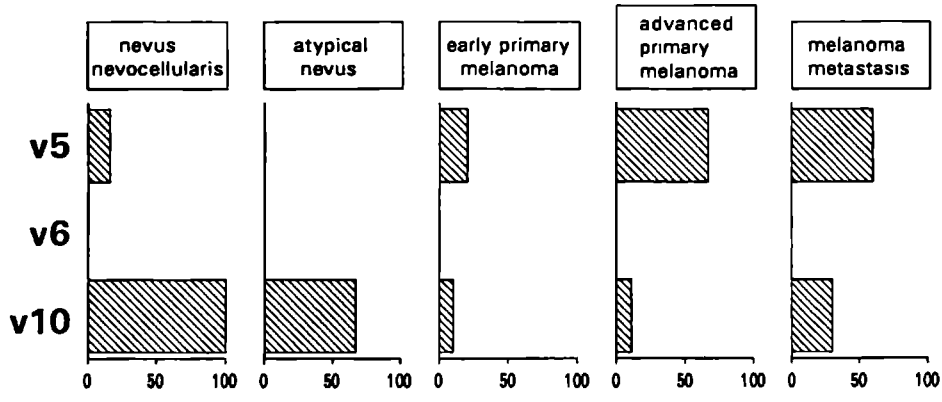


Figure 2. Expression of CD44v5 (VFF8), CD44v6 (VFF7), and CD44v10 (VFF14) in human melanocytic tumor progression. Bars indicate the percentage of positive tumors.

Interestingly, there was a marked difference in the expression of v5 and v10 in the different types of melanocytic lesions. In the lesions that were positive for v5 or v10, a random heterogeneous staining pattern was observed with 25-100% positive melanocytic cells.

V5 was detected in only a low percentage of the common and atypical nevi, and early primary melanomas, i.e. in 3/19 (16%), 0/9 (0%), and 2/10 (20%), respectively (Figs 2,3). However, the majority of the advanced primary melanomas (6/9, 67%), and melanoma metastases (11/19, 58%) expressed CD44v5 (Fig. 2,3). Hence, overexpression of v5 containing CD44 isoforms is strongly related to tumor progression ($p=0.0005$).

V10 encoded epitopes were also differentially expressed in the various types of melanocytic lesions. The pattern of expression of v10, however, was strikingly different from that of v5. V10 was detected in 100% of common nevi (19/19), in 67% of atypical nevi (6/9), in 10% of early primary melanomas (1/10), in 11% of advanced primary melanomas (1/9) and in 32% of the melanoma metastases (6/19) (Figs 2,3). Hence, malignant transformation of melanocytes is often associated with a loss of exon v10 expression ($p=0.026$).

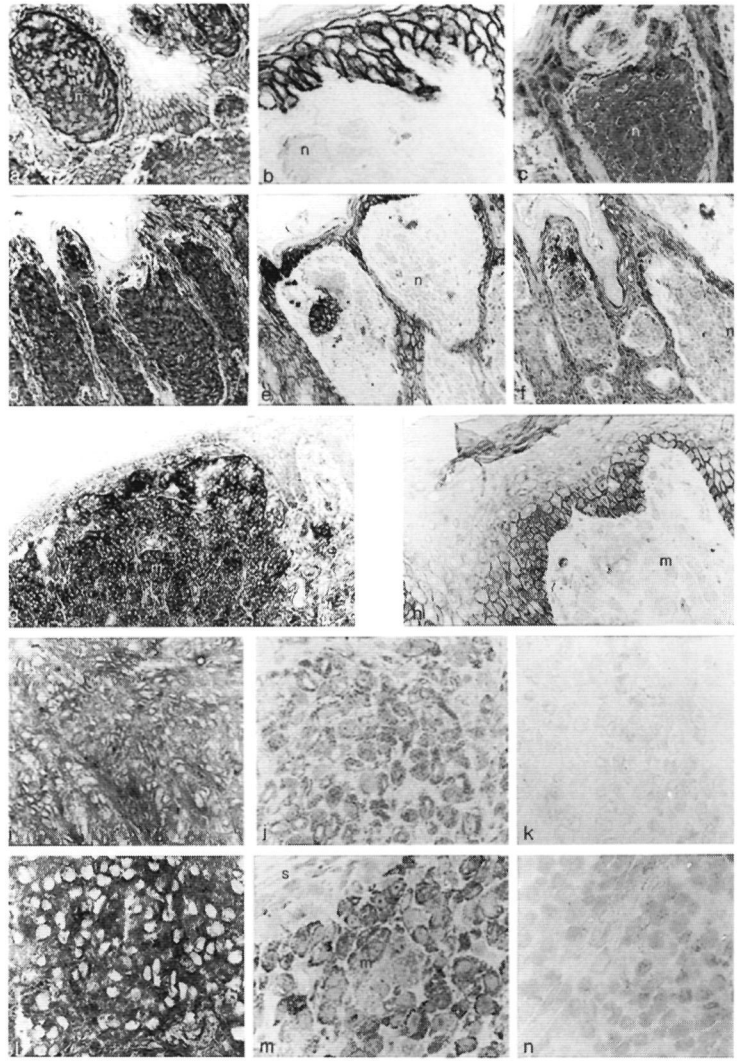


Figure 3. *Expression of CD44v on human melanocytic lesions. n=nevus cells, m=melanoma cells, s=stroma.* ***A:*** *Common melanocytic nevus strongly positive for CD44s.* ***B&C:*** *Comparable region of the same lesion as in A negative for CD44v5 (VFF8) (**B**) and positive for CD44v10 (VFF14) (**C**).* ***D:*** *Atypical nevus strongly positive for CD44s.* ***E&F:*** *Comparable region of the same lesion as in D negative for CD44v5 (VFF8) (**E**) and positive for CD44v10 (VFF14) (**F**).* ***G&H:*** *Early primary melanoma strongly positive for CD44s (**G**) and negative for CD44v5 (VFF8) (**H**).* ***I:*** *Advanced primary melanoma strongly positive for CD44s.* ***J&K:*** *Comparable region from the same lesion as in I positive for CD44v5 (VFF8) (**J**) and negative for CD44v10 (VFF14) (**K**).* ***L:*** *Melanoma metastasis strongly positive for CD44s.* ***M&N:*** *Comparable region from the same lesion as in L positive for CD44v5 (VFF8) (**M**), and negative for CD44v10 (VFF14) (**N**).*

Expression of CD44 isoforms on cultured melanocytes and melanoma cell lines

We next assessed the expression of CD44 isoforms on cultured normal melanocytes isolated from human foreskin as well as on a panel of human melanoma cell lines that are non- (IF6, 530), sporadically- (M14, Mel57), and highly (BLM, MV3) metastatic after s.c. inoculation into nude mice. Cultured normal melanocytes were found to express high levels of standard CD44 with a mean relative fluorescence intensity of approximately 5.10^3 (not shown). Epitopes encoded by v5, v6, and v10 were hardly or not detectable on melanocytes (Fig 4).

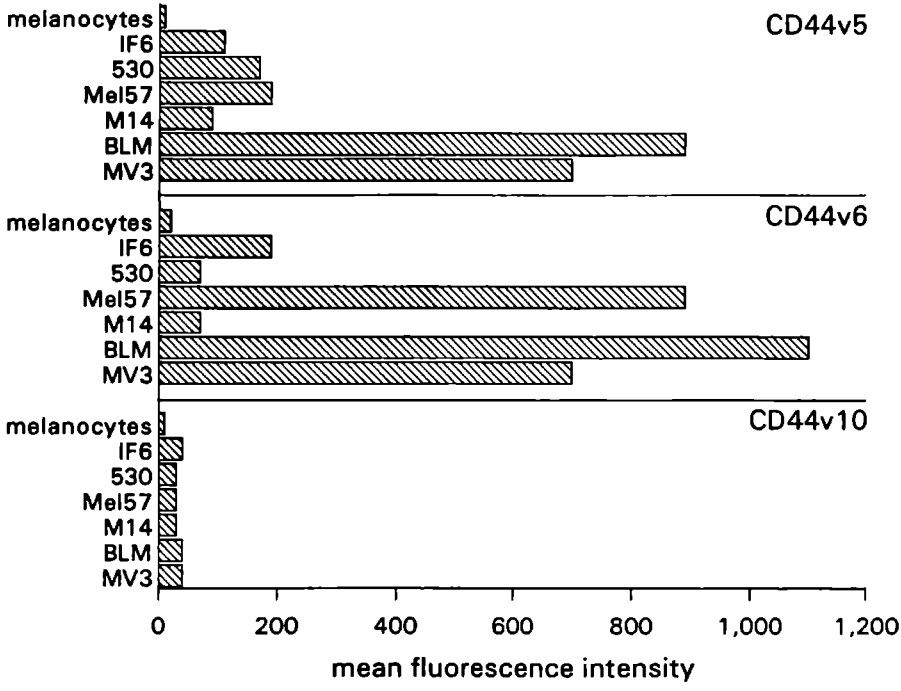


Figure 4. Expression of CD44v5 (VFF8), CD44v6 (VFF7), and CD44v10 (VFF14) on cultured melanocytes and melanoma cell lines which are non- (IF6, 530), sporadically- (M14, Mel57), or frequently (BLM, MV3) metastatic. Binding of different mAbs was measured by FACS. Results show mean fluorescence intensity. Negative controls have been subtracted.

The human melanoma cell lines also showed a strong expression of standard CD44 epitopes (not shown). This expression of CD44s was independent of the metastatic phenotype of the cell lines. Interestingly, however, expression of v5 and v6 containing

CD44 splice variants was variable and expression of v6 and particularly v5 related to the metastatic potential of the cell lines (Fig 4). Thus, expression of CD44v6 in the highly metastatic cell lines was much higher than in the non-metastatic cell lines. High expression of CD44v6 was also observed in one of the sporadically metastatic lines (i.e. MeL57). Furthermore, CD44v5 was weakly expressed on the non- and sporadically metastatic cell lines, but expression on the highly metastatic cell lines was 5-10 fold higher. All melanoma cell lines very weakly expressed CD44v10.

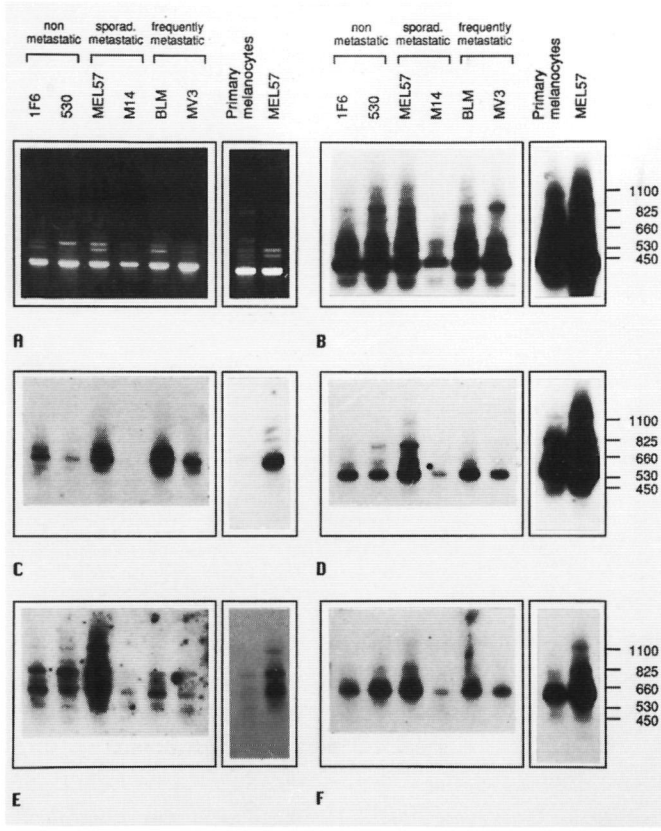


Figure 5. *Southern blot analysis of RT-PCR amplification products from specimens of cultured melanocytes and non-metastatic, sporadically metastatic, and frequently metastatic human melanoma cell lines.* ***A:*** *PCR products obtained with CD44 specific primers 5' and 3' of the variant part, were resolved on 2% agarose containing ethidium bromide, and visualized under UV-light. The bright 450 band present in all melanoma cell lines and in the cultured melanocytes corresponds to the expected standard CD44 amplification product. Expression of several larger splice variants is seen in the cultured melanocytes and all cell lines.* ***B-F:*** *After transfer of the PCR products to a Hybond N+ membrane the same filter was hybridized consecutively to* ***B:*** *standard CD44;* ***C:*** *exon v5;* ***D:*** *exon v6;* ***E:*** *exon v7; and* ***F:*** *exon v10 CD44 specific probes.*

RT-PCR analysis demonstrates multiple CD44 splice variants

To gain insight into the diversity and structure of the CD44 splice variants expressed in human melanocytes and melanoma cell lines, RNA prepared from these cells was subjected to RT-PCR amplification using primers corresponding to sequences of the 5' and the 3' standard constant region of CD44. With these primers a major PCR product of approximately 450 basepairs was obtained from the normal melanocytes as well as from the melanoma cell lines (Fig 5a). The size of this product corresponds to that expected for the standard CD44 message. In addition, up to 6 minor bands of approximately 530, 580, 615, 660, 850, and 1100 base pairs were obtained from the melanoma cell lines. Human cultured melanocytes gave rise to 5 minor bands of 530, 580, 660, 850, and 1100 base pairs (Fig 5a).

Hybridization of the PCR products of melanocytes and melanoma cell lines with specific oligonucleotide probes (Fig 5b-f), showed that all contain one to several splice variants with v6, v7, and v10. Except for M14, all melanoma cell lines contained splice variants with v5 whereas melanocytes did not hybridize with v5 specific probes. In general, the patterns obtained with the different cell lines were very similar.

DISCUSSION

Adhesion molecules mediating cell-cell or cell-matrix interactions are involved in several steps of the metastatic cascade. In malignant melanoma, increased expression of integrins $\alpha 2\beta 1$ [4,21], $\alpha 3\beta 1$ [26], $\alpha 5\beta 1$ [6], and $\alpha v\beta 3$ [1], and of ICAM1 [19], and decrease of integrin $\alpha 6\beta 1$ [27] have been reported to correlate with tumor progression. Furthermore, functional studies implicate integrin $\alpha 5\beta 1$ and $\alpha v\beta 3$ in melanoma cell proliferation [10,25] and invasion [31,32].

Several studies show a correlation between expression of members of the CD44 family of adhesion molecules and tumor progression in human non-Hodgkin lymphoma [16,17,29] and carcinoma [15,18,23,36,42]. For human melanoma, a correlation has been reported in sublines from a single cell line between high CD44 expression and metastatic property [3] but in a large panel of different human melanoma cell lines such a correlation was not confirmed [8]. In addition, CD44 is strongly expressed in all stages of human melanocytic tumor progression in vivo [24]. Our finding of comparable levels of CD44s on the surface of normal human melanocytes and melanoma cell lines with different metastatic capacities, confirms earlier findings that CD44 expression does not correlate with metastatic capacity of melanoma cells [8]. Moreover, in line with previous reports [24] we also find that CD44s is strongly expressed in all stages of melanocytic tumor progression in vivo. Hence, expression of CD44s seems to be independent of the presence of a malignant or metastatic phenotype. However, functional studies have provided evidence for a role for CD44 in human melanoma cell motility [9,37] and

invasion [9] and we have recently found that a combination of high production of hyaluronate and expression of "active" CD44, mediating adhesion to hyaluronate, correlates with metastatic capacity of human melanoma cell lines [40].

Currently, no evidence exists for the presence of CD44 splice variants in human melanoma. Therefore, in the present study we have investigated expression of CD44 variants, 1) in lesions representing various stages of human melanocytic tumor progression in situ, and 2) in a panel of human melanoma cell lines with different metastatic potential after s.c. inoculation into nude mice.

In contrast to CD44s, CD44 isoforms are differentially expressed. Expression of CD44 isoforms containing v5, is not found on normal human melanocytes but v5 is expressed on human melanoma cell lines and its expression is strongly enhanced in the highly metastatic cell lines. In addition, v5 is expressed in a very low percentage of benign melanocytic lesions and early primary melanomas, whereas it is expressed in the majority of advanced primary melanomas and melanoma metastases. Thus, enhanced expression of CD44v5 is related to high metastatic potential of human melanoma cell lines and correlates with human melanocytic tumor progression in vivo. Interestingly, CD44v5 is also induced in human colorectal tumor progression [42]. Similar to this type of cancer where v5 is already expressed in early stages of adenoma, v5 can also be detected in a few benign melanocytic nevi. However, in contrast to colorectal tumor progression, the transition from early primary melanoma to the highly aggressive stage of advanced primary melanoma, is attended by a dramatic increase of the percentage of v5 positive lesions. It is noteworthy that gastric diffuse type adenocarcinomas and their metastases also express CD44v5 and that they are devoid of v6 as well [14].

CD44v6 containing isoforms have been related to tumor progression in human colorectal carcinomas and malignant lymphomas [22,42]. Moreover, v6 containing isoforms have been shown to play a causal role in metastasis of rat pancreatic carcinoma cells [11,33]. However, by immunohistochemical staining of malignant melanomas and melanoma metastases, we do not detect any expression of CD44v6. This lack of CD44v6 expression contrasts with the results obtained in the six human melanoma cell lines, which all express v6. At present, we have no explanation for the discrepancy in CD44v6 expression between cultured cell lines and fresh human malignant melanomas. Expression of v6 on the melanoma cell lines may be induced by the in vitro culture conditions. Alternatively, the v6 epitope might be masked by ligand binding or alterations in the conformation of the CD44 molecule in situ.

In complete contrast to expression of v5, we find that expression of CD44v10 is inversely correlated to melanocytic tumor progression in vivo. Most metastases lack expression of this variant and in line with this finding, a very low surface expression of CD44v10 is found on all melanoma cell lines tested, which all were originally generated from metastases.

At the RNA level, the patterns of CD44 splice variant expression are closely similar

in the various cell lines. This finding is remarkable since the cell lines studied are mutually unrelated. Hence, the splicing patterns observed do not represent idiosyncratic changes of an individual clone but may represent a tissue- or tumor-type specific quality.

At present, the functional consequence of insertion of various exons into the CD44 molecule remains unknown. It may be speculated that the ligand binding specificity is altered. The 90 kDa CD44s isoform (CD44H) that is expressed on most cells, binds hyaluronate [2]. For hyaluronate binding of CD44E, an isoform that contains a 132 amino-acid inserted domain, the data are conflicting [13,35]. Our cell lines, including melanocytes, all express the 90 kDa CD44s molecule [40] and adhesion to hyaluronate observed for melanocytes, IF6, Mel57, BLM and MV3 [40] is probably via this isoform. Therefore, in this system we cannot look into the role of the splice variants in attachment to hyaluronate. Further studies should provide more insight in this matter.

Taken together, we observed that an increase of CD44v5 and a decrease of CD44v10 expression is related to melanocytic tumor progression in vivo and that enhanced expression of CD44v5 is related to the metastatic capacity of melanoma cell lines. These findings may suggest a role for CD44v5 and CD44v10 in human melanocytic tumor progression and melanoma metastasis.

ACKNOWLEDGEMENTS

We thank H. van Vught and K. Jansen for expert technical assistance, Dr. CG. Figdor (Nijmegen, The Netherlands) for mAb NKI-P1, Drs. G. Adolf and E. Patzelt (Vienna, Austria) for the VFF-series of mAbs, Dr. E-B. Bröcker (Würzburg, Germany) for providing some of the patients lesions, and Drs. F. van de Berg and P. Das (Amsterdam, The Netherlands) and P. Herrlich (Karlsruhe, Germany) for helpful discussions. This work was supported by the Dutch Cancer Society (grant NUKC 91-09).

REFERENCES

1. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M. Integrin distribution in malignant melanoma: association of the $\beta 3$ subunit with tumor progression. Cancer Res 50, 6757-6764, 1990.

2. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 61, 1303-1313, 1990.

3. Birch M, Mitchel S, Hart IR. Isolation and characterization of human melanoma variants expressing high and low levels of CD44. Cancer Res 51, 6660-6667, 1991.

4. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C. Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36, 29-35, 1985.

5. Brüggen J, Sorg C, Macher E. Membrane-associated antigens of human malignant melanoma: serological

typing of cell lines using antisera from non-human primates. Cancer Immunol Immunother 5, 53-68, 1978.
6. Danen EHJ, Ten Berge PJM, Van Muijen GNP, Van 't Hof-Grootenboer AE, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin receptor in melanocytic tumor progression. Histopathol 24, 249-256, 1994
7. Danen EHJ, Van Muijen GNP, van de Wiel-van Kemenade E, Jansen KFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and non-metastatic and highly metastatic human melanoma cells Int J Cancer 54, 315-321, 1993.
8. East JA, Mitchell SD, Hart IR. Expression and function of the CD44 glycoprotein in melanoma cell lines. Melanoma Res 3, 341-346, 1993.
9. Faassen AE, Schrager JA, Klein DJ, Oegema TR, Couchman JR, McCarthy JB. A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J Cell Biol 116, 521-531, 1992.
10. Felding-Habermann B, Müller BM, Romerdahl CA, Cheresh DA. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J Clin Invest 89, 2018-2022, 1992.
11. Günthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussman I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells Cell 65, 13-24, 1991.
12. Hart IR, Goode NT, Wilson RE. Molecular aspects of the metastatic cascade. Biochim Biophys Acta 65-84, 1989.
13. He Q, Lesley J, Hyman R, Ishihara K, Kincade PW. Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. J Cell Biol 119, 1711-1719, 1992.
14. Heider K-H, Dammrich J, Skroch-Angel P, Müller-Hermelink HK, Vollmers HP, Herrlich P, Ponta H. Differential expression of CD44 splice variants in intestinal and diffuse type human gastric carcinomas and normal gastric mucosa. Cancer Res 53, 4197-4203, 1993.
15. Heider K-H, Hofmann M, Horst E, van den Berg F, Ponta H, Herrlich P, Pals ST. A human homologue of rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. J Cell Biol 120, 227-233, 1993.
16. Horst E, Meijer CJLM, Radaszkiewicz T, Ossekoppele GJ, van Krieken JHJM, Pals ST. Adhesion molecules in the prognosis of diffuse large cell lymphoma: expression of a lymphocyte homing receptor CD44, LFA-1 (CD11a/CD18), and ICAM-1 (CD54). Leukemia 4, 595-599, 1991.
17. Jalkanen S, Joensuu H, Klemi P. Prognostic value of lymphocyte homing receptor and S value in Non Hodgkin's Lymphoma. Blood 75, 1549-1556, 1990.
18. Joensuu H, Klemi PJ, Toikkanen S, Jalkanen S. Glycoprotein CD44 expression and its association with survival in breast cancer. Am J Pathol 143, 867-875, 1993.
19. Johnson JP, Stade BG, Holzmann B, Schwalbe W, Rietmüller G. De novo expression of intercellular adhesion molecule-1 in melanoma correlates with increased risk of metastasis. Proc Natl Acad Sci USA 86, 641-644, 1989.
20. Katano M, Saxton RE, Cochran AJ, Irie RF. Establishment of an ascitic human melanoma cell line that metastasizes to lung and liver in nude mice. J Clin Cancer Res Clin Oncol 108, 197-203, 1984.
21. Klein CE, Steinmeyer T, Kaufmann D, Weber L, Bröcker EB. Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96, 281-284, 1984.
22. Koopman G, Heider K-H, Horst E, Adolf GR, van den Berg F, Ponta H, Herrlich P, Pals ST. Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 177, 897-904, 1993
23. Mayer B, Jauch KW, Günthert U, Figdor CG, Schildberg FW, Funke I, Johnson JP. De-novo expres-

sion of CD44 and survival in gastric cancer. Lancet 342, 1019-1022, 1993.
24. Moretti S, Martini L, Berti E, Pinzi C, Gianotti B. Adhesion molecule profile and malignancy of melanocytic lesions. Melanoma Res 3, 235-239, 1993.
25. Mortarini R, Gismondi A, Santoni A, Parmiani G, Anichini A. Role of the $\alpha 5\beta 1$ integrin in the proliferative response of quiescent human melanoma cells to fibronectin. Cancer Res 52, 1919-1929, 1992.
26. Natali PG, Nicotra MR, Bartolazzi A, Cavaliere R, Bigotti A. Integrin expression in cutaneous malignant melanoma: association of the $\alpha 3\beta 1$ heterodimer with tumor progression. Int J Cancer 54, 68-72, 1993.
27. Natali PG, Nicotra MR, Cavaliere R, Giannarelli D, Bigotti A. Tumor progression in human malignant melanoma is associated with changes in $\alpha 6\beta 1$ laminin receptor. Int J Cancer 49, 168-172, 1991.
28. Pals ST, Hogervorst F, Keizer GD, Thepen T, Horst E, Figdor CG. Identification of a widely distributed 90kD glycoprotein that is homologous to the Hermes-1 human lymphocyte homing receptor. J Immunol 143, 851-857, 1989.
29. Pals ST, Horst E, Ossekoppele GJ, Figdor CG, Scheper RJ, Meijer CJLM. Expression of lymphocyte homing receptor as a mechanism of dissemination in non Hodgkin's Lymphomas. Blood 73, 995-998, 1989.
30. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Geth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci USA 89, 12160-12164, 1992.
31. Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the $\alpha v\beta 3$ integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 89, 1557-1561, 1992.
32. Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC. The 72 kDa type IV collagenase is modulated via differential expression of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins during human melanoma cell invasion. Cancer Res 53, 3411-3415, 1993.
33. Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzku S, Zoller M. Prevention of tumor metastasis formation by anti-variant CD44. J Exp Med 177, 443-455, 1993.
34. Smit NPM, Westerhof W, Asghar SS, Pavel S, Siddiqui AH. Large-scale cultivation of human melanocytes using collagen-coated sephadex beads (cytodex 3). J Invest Dermatol 92, 18-21, 1989.
35. Stamenkovic I, Aruffo A, Amiot M, Seed B. Hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate bearing cells. EMBO J 10, 343-348, 1991.
36. Tanabe K, Ellis LM, Saya H. Expression of CD44R1 adhesion molecule in colon carcinoma and metastasis. Lancet 341, 725-7261, 1993.
37. Thomas L, Byers HR, Vink J, Stamenkovic I. CD44H regulates tumor cell migration on hyaluronate-coated substrate. J Cell Biol 118, 971-977, 1992.
38. Van Muijen GNP, Cornelissen IMHA, Jansen CFJ, Figdor CG, Johnson JP, Bröcker EB, Ruiter DJ. Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin Exp Metastasis 9, 259-272, 1991.
39. Van Muijen GNP, Jansen CFJ, Cornelissen IMHA, Smeets DFCM, Beck JLM, Ruiter DJ. Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice Int J Cancer 48, 85-91, 1991.
40. Van Muijen GNP, Danen EHJ, Veerkamp JH, Ruiter DJ, Lesley J, Van de Heuvel LPWJ. Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity. Int J Cancer 61, 241-248, 1995.
41. Versteeg R, Noordermeer IA, Kruse-Wolters M, Ruiter DJ, Schrier PI. C-myc down-regulates class I HLA expression in human melanomas. EMBO J 7, 1023-1029, 1988.
42. Wielenga VJM, Heider K-H, Offerhaus GJA, Adolf GR, van den Berg FM, Ponta H, Herrlich P, Pals ST. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression Cancer Res 53, 4754-4756, 1993.

Expression of CD44 and the patern of CD44 alternative splicing in uveal melanoma

Melanoma Res, **in press**

Expression of CD44 and the patern of CD44 alternative splicing in uveal melanoma

Erik HJ Danen[1], Paul JM ten Berge[2], Goos NP van Muijen[1], Martine J Jager[3], and Dirk J Ruiter[1]

[1]Department of Pathology, University Hospital, Nijmegen, [2]Department of Ophthalmology, University Hospital, Maastricht, and [3]Department of Ophthalmology, University Hospital, Leiden, The Netherlands

In cutaneous melanoma, the standard CD44 molecule is abundantly expressed whereas expression of certain splice variants is related to tumor progression and to the metastatic potential of cell lines. In the present study we explored the expression of CD44 and the pattern of CD44 alternative splicing in uveal melanoma, in relation to cell type, diameter, and invasiveness of the tumor. All uveal melanomas strongly stained with antibodies to the standard portion of CD44. No expression was found of CD44v7 whereas v5, v6, and v10 were expressed (2/12, 5/12, and 8/12 respectively). No correlation was observed between expression of certain splice variants and cell type, tumor diameter, or invasion of the sclera or Bruch's membrane. All three uveal melanoma cell lines tested were strongly CD44 positive and expressed low levels of v6 containing isoforms at the cell surface, whereas CD44v5, v7, and v10 were absent. Our results show that CD44 is strongly expressed in uveal melanoma and that the pattern of CD44 alternative splicing is similar to that observed in cutaneous melanoma. However, in contrast to cutaneous melanoma it does not seem to be related to prognostic parameters in uveal melanoma.

INTRODUCTION

Changes in the adhesive behavior of cells are considered to be important events in the process of tumor progression. Cells can bind to the extracellular matrix and to other cells via members of the integrin-, selectin-, cadherin-, immunoglobulin- or CD44 families of adhesion receptors [11].

CD44 is a family of transmembrane glycoproteins derived from one single gene whose members differ structurally as a result of different glycosilation and alternative exon splicing [15]. The standard 80-90 kDa CD44 molecule is expressed on most cell types and mediates adhesion and migration on hyaluronate [1,24]. For the larger isoforms, generated by alternative splicing of 10 exons that are expressed mainly on epithelial cells, data for hyaluronate binding are conflicting [*see* 15]. The expression pattern of CD44 isoforms changes during development and differentiation [19], and is altered with tumor progression in non-Hodgkin's lymphoma [13], colon carcinoma [10,26], gastric carcinoma [9], and cervical carcinoma [3]. A causal role in metastasis of rat prostate carcinoma cells has been demonstrated for isoforms containing the variant exon v6 product by transfection [7] and antibody blocking studies [21].

CD44-mediated hyaluronate binding may play a role in tumorigenesis and metastasis of human cutaneous melanoma. Transfection of a CD44 negative melanoma cell line with cDNA for the 80-90 kDa CD44 molecule, promotes tumorigenicity [2]. In addition, growth and metastasis of melanoma cells can be inhibited by antibodies to CD44 [8]. However, expression of CD44 is not related to the metastatic potential of melanoma cells [6,25], and CD44 is strongly expressed in all stages of melanocytic tumor progression in situ [16,17]. We recently reported that expression of CD44v5 is related to melanocytic tumor progression in situ and to the metastatic potential of cell lines in nude mice [16].

Melanomas not only originate from melanocytes in the skin, but also from melanocytes in the eye, i.e. uveal melanoma. For both types of melanoma, prognosis is influenced by the tumor diameter, but in uveal melanoma the cell type (epithelial versus spindle) is an additional important parameter. Furthermore, in contrast to cutaneous melanoma, uveal melanoma metastasizes primarily to the liver, and as the eye lacks lymphatics they do not lymphatically spread. As uveal melanoma thus differs biologically and clinically from cutaneous melanoma, uveal melanoma may differ in expression of CD44 and in the pattern of CD44 alternative splicing.

MATERIAL AND METHODS

Antibodies

The mAb directed to the constant part of the CD44 molecule was NKI-P2 [18], provided by Dr. CG. Figdor (Nijmegen, The Netherlands). MAbs directed to the variant

portions of CD44 were VFF8 anti-CD44v5 (IgG1), VFF7 anti-CD44v6 (IgG1), VFF9 anti-CD44v7 (IgG1), and VFF14 anti-CD44v10 (IgG1), purchased from Bender MedSystems (Vienna, Austria), and VFF4 anti-CD44v6 (IgG2b), provided by Dr. ST. Pals (Amsterdam, The Netherlands) [13,26]. WT31 anti-CD3 [22] (IgG1), provided by Dr. W. Tax (Nijmegen, The Netherlands), was used as a control antibody in flowcytometrical analyses.

Immunohistochemistry

Specimens of 12 primary uveal melanomas were obtained from patients at the University Hospitals in Nijmegen and Leiden, The Netherlands. Diagnosis of primary uveal melanomas was microscopically assessed on paraffin sections, discerning spindle (n=3), mixed (n=7) and epithelioid (n=2) cell types. Invasion of the sclera was graded as not (n=2), superficially (ca. 25% of scleral thickness) (n=3), half (ca. 50% of scleral thickness) (n=3), deep (ca. 75% of scleral thickness) (n=1), and episcleral growth (n=3). Bruch's membrane was regarded as intact (n=2) or ruptured (n=8).

Representative parts of all specimens were embedded in Tissue Tek OCT compound (Ames Company, Division of Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen. In a cryostat 4μm sections were cut at -25°C, mounted on uncoated slides and stored at -80°C until use. Sections were air dried, fixed in acetone for 10 min, and incubated with the mAbs at room temperature for 1 h. MAbs were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA); optimal working dilutions had been previously determined on positive controls (epidermis of normal skin). After PBS rinsing, detection of the primary antibodies was achieved using the peroxidase-based Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). This consisted of a biotinylated horse anti-mouse IgG, followed by an avidin-biotin-peroxidase complex (ABC). As a substrate, 3-amino-9-ethyl-carbazole (AEC) in acetate buffer pH 4.85 containing hydrogen peroxide was used. Finally, sections were counterstained with hematoxylin and mounted with Kaiser's glycerol/gelatin (Merck, Darmstadt, Germany). Negative controls consisted of incubations replacing the primary antibodies by PBS/BSA.

The percentage of stained melanocytic cells was estimated as: 0, 1-25, 26-50, 51-75, 76-100%. Slides were read independently by two observers. Discrepancies exceeding one percentage class were found in less than 10% of the cases. These cases were reevaluated jointly until consensus was reached.

Cell lines and culture conditions

The human uveal melanoma cell lines Mel202 [14], provided by Dr. BR. Ksander (Boston, MA); 92-I [5], provided by Dr. I. de Waard-Siebinga (Leiden, The Netherlands); and OCM-1 [12], provided by Dr. GPM. Luyten (Rotterdam, The Netherlands); were studied for expression of CD44 splice variants. The cell lines were grown in DMEM (Flow, Irvine, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum

(Hyclone, Logan, UT) and antibiotics.

Flowcytometry

Flowcytometry was done as described before [4]. In short, cells were sequentially incubated in PBS containing 1% BSA and 0.02% sodium azide with appropriate dilutions of the various mAbs, followed by fluorescein-isothiocyanate (FITC) conjugated F(ab')2 fragments of rabbit anti-mouse Ig (Dako, Glostrup, Denmark). Analyses were performed on an Epics Elite flowcytometer (Coulter, Mijdrecht, The Netherlands).

Table 1. Expression of CD44 splice variants in uveal melanoma lesions.

	cell type	diam. (mm)	sclera (% invasion)	Bruchs	CD44s	CD44v5	CD44v6	CD44v7	CD44v10
					(% positive cells)				
1	*mixed*	*19*	*100*	*rupt.*	100	0	0	0	0
2	*spindle*	*13.5*	*75*	*rupt.*	100	0	0	0	0
3	*mixed*	*11*	*50*	*intact*	100	0	0	0	75-100
4	*epith.**	*2*	*25*	*n.i.*	100	1-5	1-5	0	75-100
5	*spindle*	*12*	*25*	*rupt.*	100	0	1-5	0	50-75
6	*mixed*	*12*	*50*	*rupt.*	100	0	5-25	0	5-25
7	*mixed*	*16*	*50*	*n.i.*	100	0	0	0	25-50
8	*spindle*	*8*	*0*	*rupt.*	100	0	0	0	0
9	*mixed*	*12*	*25*	*intact*	100	0	5-25	0	100
10	*mixed*	*11*	*0*	*rupt.*	100	0	0	0	0
11	*mixed*	*11*	*100*	*rupt.*	100	1-5	1-5	0	75-100
12	*epith.*	*16*	*100*	*rupt.*	100	0	0	0	50-75

*epith.=epithelioid; diam.=diameter; rupt.=ruptured; n.i.=not identifiable.

RESULTS

Expression of CD44 splice variants in uveal melanoma lesions

By immunohistochemistry we found differential expression of the various CD44 epitopes in uveal melanoma (Table 1, Fig 1). A strong and homogeneous expression was observed in all uveal melanomas with NKI-P2 (Fig 1a) directed against an epitope on the NH_2- terminal constant portion of CD44. The quality of the mAbs to CD44v regions was tested on normal skin where all mAbs stained keratinocytes (not shown). In addition, as an internal control, retinal cells present in some of the lesions stained with all mAbs used (not shown). Expression of CD44v10 (Fig 1d) could be detected in 8/12 lesions and in most positive lesions more than 50% of the tumor cells were positive. CD44v6 was detected in 5/12 lesions with VFF4 (Fig 1c) and 2 of those lesions also showed a positive

reaction with VFF7 anti-CD44v6. Only 2/12 uveal melanomas were found to be positive for CD44v5 (Fig 1b). Lesions that were positive for CD44v5 or CD44v6 showed expression on less then 25% of the tumor cells. Expression of CD44v7 was not detected in any of the lesions. Expression of CD44 splice variants showed no relation to tumor diameter, cell type, scleral infiltration, or Bruch's membrane invasion.

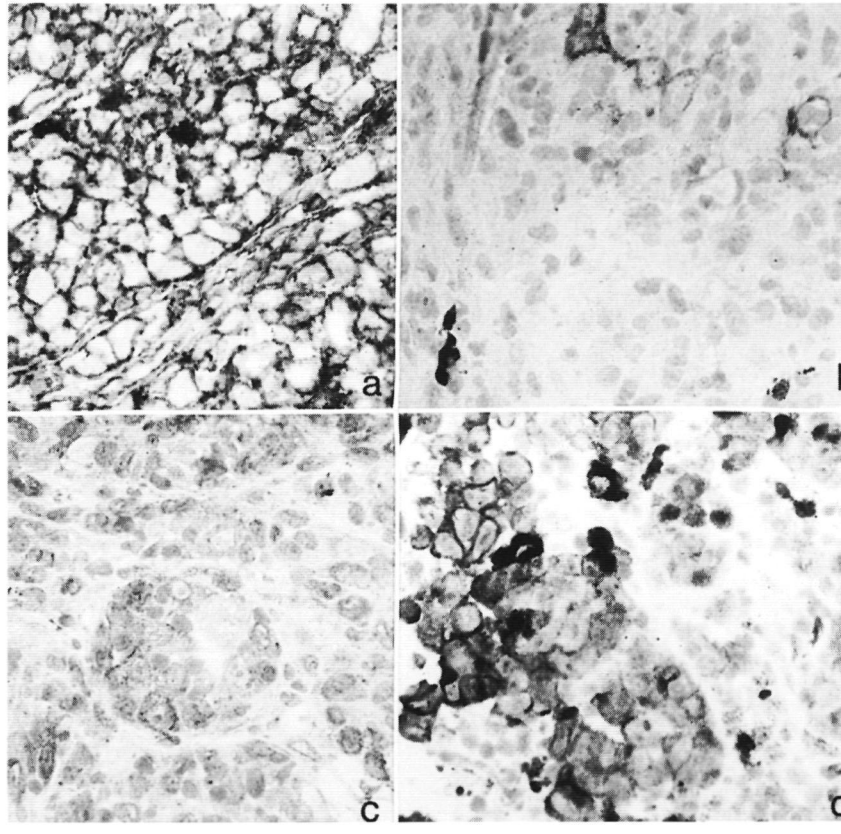


Figure 1. *Immunohistochemical staining for CD44 splice variants on uveal melanoma lesions. Frozen sections of uveal melanomas were stained with NKI-P2 directed against the constant CD44 portion* ***(A)****, VFF8 anti-CD44v5* ***(B)****, VFF4 anti-CD44v6* ***(C)****, or VFF14 anti-CD44v10* ***(D)****.*

Expression of CD44 splice variants on uveal melanoma cell lines

All 3 uveal melanoma cell lines expressed high levels of CD44 at their surface as detected by NKI-P2 (Fig 2). No expression of CD44v5, CD44v7 or CD44v10 was observed on any of the cell lines, while CD44v6 was detectable on all 3 cell lines by 2 CD44v6 mAbs. Expression of CD44v6 was low in comparison to the level of staining with NKI-P2, showing that only a minor portion of the abundantly expressed CD44 molecules, contains the v6 exon product.

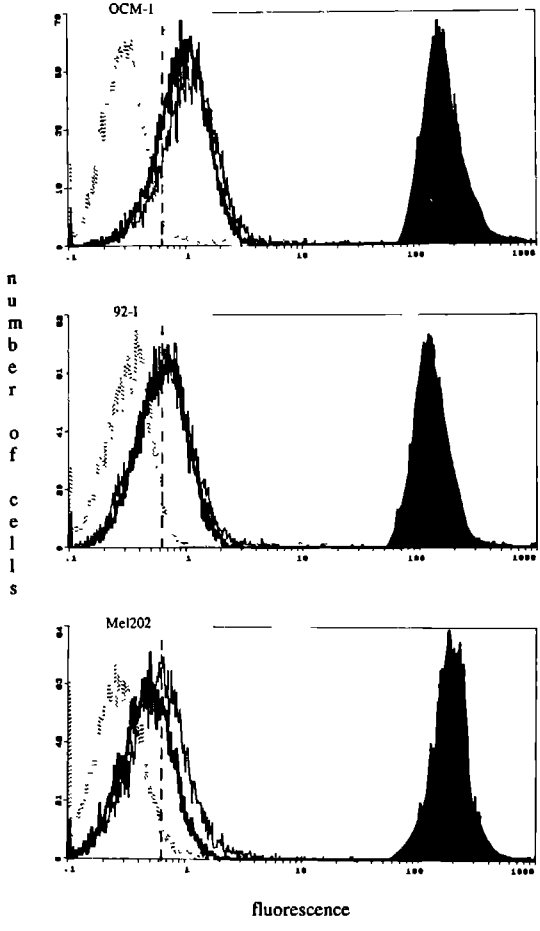


Figure 2. Surface expression of CD44v6 on uveal melanoma cell lines. Three uveal melanoma cell lines were stained with VFF4 (thin graph) or VFF7 (thick graph) anti-CD44v6 mAbs. Staining with the control anti-CD3 mAb is indicated by the dotted graph. Incubation with mAbs to CD44v5 or CD44v10 gave a similar result as anti-CD3 (not shown). Strong staining was observed with NKI-P2 against a standard epitope and recognizing all CD44 isoforms (filled graph).

Our finding that staining with the NKI-P2 mAb directed to the standard portion of CD44, is very strong on all uveal melanomas, is in line with findings for cutaneous melanomas [16,17]. This mAb detects all CD44 isoforms. The major species detected with this mAb on both types of melanoma is probably the standard 80-90 kDa CD44 molecule. Also, on the uveal melanoma cell lines, it is most likely that the very high expression observed with this mAb, represents predominantly the standard CD44, as it does on cell lines derived from cutaneous melanoma [25].

Our data show that the CD44 splice variants that can be detected in cutaneous melanoma, are also found in uveal melanoma. We find CD44v10 expression in uveal melanoma lesions but not on uveal melanoma cell lines, which agrees with the observation that CD44v10 is absent or present at a very low level on the surface of cell lines derived from cutaneous melanoma whereas cutaneous melanoma lesions can have strong expression of v10 in situ [16]. CD44v6 can be detected on cutaneous melanoma cell lines [16] and we find v6 expression on each of the uveal melanoma cell lines investigated. However, in contrast to the fact that CD44v6 cannot be detected in fresh lesions in any stage of cutaneous melanocytic tumor progression in situ [16,20], in the present study we find that 5/12 uveal melanomas express this variant. The fact that CD44v6 expression is induced on cutaneous melanoma cells by culturing them, and the marked difference between fresh cutaneous and uveal melanomas with respect to v6 expression, may suggest that the microenvironment of the melanoma cells influences expression of this variant.

For CD44v5 the opposite pattern is observed. While the majority of the primary cutaneous melanomas express CD44v5 [16], only 2 of the 12 uveal melanomas show v5 expression. Furthermore, in these two lesions only a small percentage of cells was positive. In cutaneous melanoma expression of v5 is found preferentially in advanced (thick) lesions which are associated with a poor prognosis. For uveal melanoma, even lesions with signs of an unfavorable prognosis as based on a large tumor diameter, mixed or epithelioid cell types, a broken Bruch's membrane, and complete scleral invasion, are negative. Thus, even though previous findings suggested a role for CD44v5 in cutaneous melanocytic tumor progression, CD44v5 seems not to be involved in uveal melanoma invasion or metastasis. We have previously reported a similar phenomenon for the $\alpha v\beta 3$ integrin [23]. Expression of this integrin is acquired in advanced stages of cutaneous melanocytic tumor progression whereas it is absent from all primary uveal melanomas. Together these findings suggest that the role of adhesion molecules in outgrowth of the primary tumor and release of tumor cells leading to distant metastases may differ for cutaneous and uveal melanoma.

In conclusion, our results demonstrate that CD44 is strongly expressed in uveal melanoma and that the pattern of CD44 alternative splicing in cutaneous and uveal melanoma is similar. In contrast to cutaneous melanoma, expression of CD44v in uveal

melanoma is not related to tumor thickness or other prognostic parameters.

ACKNOWLEDGEMENTS

We thank Drs. Carl Figdor, Steven Pals, and Wil Tax for generously providing the antibodies, and Drs. B. Ksander, Gré Luyten, and Itte de Waard-Siebinga for providing the cell lines. This study was supported by the Dutch Cancer Society (grant NUKC-9109).

REFERENCES

1. Arrufo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 61, 1303-1313, 1990.

2. Bartolazzi A, Peach R, Aruffo A, Stamenkovic I. Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. J Exp Med 180, 53-66, 1994.

3. Dall P, Heider K-H, Hekele S, von Minckwitz G, Kaufmann M, Ponta H, Herrlich P. Surface protein expression and messenger RNA-splicing analysis of CD44 in uterine cervical and normal cervical epithelium. Cancer Res 54, 3337-3341, 1994.

4. Danen EHJ, Van Muijen GNP, van de Wiel-van Kemenade E, Jansen KFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and non-metastatic and highly metastatic human melanoma cells. Int J Cancer 54, 315-321, 1993.

5. De Waard-Siebinga I, Blom D-JR, Griffioen M, Schrier PI, Hoogendoorn E, Beverstock G, Danen EHJ, Jager MJ. Establishment and characterization of a uveal melanoma cell line. Int J Cancer 62, 155-166, 1995.

6. East JA, Mitchell SD, Hart IR. Expression and function of the CD44 glycoprotein in melanoma cell lines. Melanoma Res 3, 341-346, 1993.

7. Günthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussman I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65, 13-24, 1991.

8. Guo Y, Ma J, Wang J, Che X, Narula J, Bigby M, Wu M, Sy M. Inhibition of human melanoma growth and metastasis in vivo by anti-CD44 monoclonal antibody. Cancer Res 54, 1561-1565, 1994.

9. Heider K-H, Dammrich J, Skroch-Angel P, Müller-Hermelink HK, Vollmers HP, Herrlich P, Ponta H. Differential expression of CD44 splice variants in intestinal and diffuse type human gastric carcinomas and normal gastric mucosa. Cancer Res 53, 4197-4203, 1993.

10. Heider K-H, Hofmann M, Horst E, van den Berg F, Ponta H, Herrlich P, Pals ST. A human homologue of rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. J Cell Biol 120, 227-233, 1993.

11. Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. Cell 68, 303-322, 1992.

12. Kan-Mitchell J, Mitchell MS, Rao N, Liggett PE. Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. Invest Ophthalmol Vis Sci 30, 829-834, 1989.

13. Koopman G, Heider K-H, Horst E, Adolf GR, van den Berg F, Ponta H, Herrlich P, Pals ST Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 177, 897-904, 1993.

14. Ksander BR, Rubsamen BE, Olsenk R, Cousius SW, Streilein JW. Studies of tumor-infiltrating

lymphocytes from a human choroidal melanoma. Invest Ophthalmol Vis Sci 32, 3198-3208, 1991

15. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix Adv Immunol 54, 271-335, 1993.

16. Manten-Horst E, Danen EHJ, Smit L, Le Poole C, Van Muijen GNP, Pals ST, Ruiter DJ Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential. Int J Cancer 64, 182-188, 1995

17. Moretti S, Martini L, Berti E, Pinzi C, Gianotti B. Adhesion molecule profile and malignancy of melanocytic lesions. Melanoma Res 3, 235-239, 1993.

18. Pals ST, Hogervorst F, Keizer GD, Thepen T, Horst E, Figdor CG. Identification of a widely distributed 90kD glycoprotein that is homologous to the Hermes-1 human lymphocyte homing receptor. J Immunol 143, 851-857, 1989.

19. Ruiz P, Schwärzler C, Günthert U. CD44 isoforms during differentiation and development. BioEssays 17, 17-24, 1995.

20. Schadendorf D, Heidel J, Gawlik C, Suter L, Czarnetzki BM Association with clinical outcome of expression of VLA-4 in primary melanoma as well as P-selectin and E-selectin on intratumoral vessels J Natl Cancer Inst 87, 366-371, 1995.

21. Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzku S, Zöller M Prevention of tumor metastasis formation by anti-variant CD44 J Exp Med 177, 443-455, 1993

22. Tax WJM, Willems HW, Reekers PPM, Capel PJA, Koene RAP Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells Nature 304, 445-447, 1983.

23. Ten Berge PJM, Danen EHJ, Van Muijen GNP, Jager M, Ruiter DJ. Integrin expression in uveal melanoma differs from cutaneous melanoma. Invest Ophthalmol Vis Sci 34, 3635-3640, 1993.

24. Thomas L, Byers RH, Vink J, Stamenkovic I. CD44H regulates tumor cell migration on hyaluronate-coated substrate. J Cell Biol 118, 971-977, 1993.

25. Van Muijen GNP, Danen EHJ, Veerkamp JH, Ruiter DJ, Lesley J, Van den Heuvel LPWJ. Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity. Int J Cancer 61, 241-248, 1995.

26. Wielenga VJM, Heider K-H, Offerhaus GJA, Adolf GR, van den Berg FM, Ponta H, Herrlich P, Pals ST. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression Cancer Res 53, 4754-4756, 1993.

Summary / Samenvatting

Summary

Metastatic spread is the major cause of mortality in cancer patients. In order to go through the steps of tumor progression and metastasize, cancer cells must have the appropriate adhesive phenotype to be able to cross basement membranes and invade tissues. Several cell adhesion receptor families are known that are involved in intercellular adhesion and attachment of cells to the extracellular matrix, i.e. cadherins, selectins, members of the immunoglobulin superfamily, integrins, and CD44 molecules. The fact that several of these can act as signaling receptors controlling cellular proliferation and differentiation, in addition to their role in adhesion and migration events, further implicates them in tumor metastasis. General aspects of the cell adhesion receptors and evidence for their role in tumor progression and metastasis are reviewed in chapter 1.

In this thesis, cell adhesion receptors are studied in human melanoma, a highly aggressive tumor originating from cutaneous or uveal melanocytes. To investigate changes in the expression of cell adhesion receptors with melanocytic tumor progression, patients lesions from the different stages in situ are studied, including benign nevi, primary melanomas, and metastases (findings for normal melanocytes in situ are difficult to interpret because of the close contact with keratinocytes). In addition, expression and function of cell adhesion receptors is investigated in cultured human melanocytes and human melanoma cell lines with different metastatic potentials in nude mice.

In chapter 2, the adhesive phenotype of cultured normal melanocytes is investigated. It is found that the level of expression of certain integrins in proliferating melanocytes differs from that in differentiated melanocytes. As a result, proliferating melanocytes lose the ability to adhere to basement membrane components. In contrast, expression of E-cadherin and binding to keratinocytes via this receptor is found for melanocytes in both conditions.

As E-cadherin on keratinocytes has been shown to be lost in invasive carcinoma, in chapter 3 the hypothesis is investigated that a similar phenomenon may occur in melanoma. The E-cadherin expression observed for cultured melanocytes and nevus cells is indeed lost in metastatic melanoma cell lines, but no expression can be detected in nevocellular nevi studied in situ. Furthermore, expression is sometimes detected in primary melanomas and metastases. Thus, E-cadherin-mediated adhesion of cultured melanocytes to keratinocytes may reflect an artefact, and loss of E-cadherin does not seem to be involved in early stages of melanocytic tumor progression.

In chapter 4, integrin expression is studied in lesions of cutaneous melanocytic tumor progression in situ. Acquired expression of $\alpha 5\beta 1$ and $\alpha v\beta 3$ in advanced stages are the major findings. As uveal melanoma differs both clinically and biologically from cutaneous melanoma, it may express other adhesion receptors. Indeed, in chapter 5, all primary uveal melanomas studied are shown to express $\alpha 5\beta 1$ while none of them express $\alpha v\beta 3$.

Adhesion to the endothelial basement membrane may be a critical event in melanoma

metastasis. This idea is supported by the finding in chapter 6, that in highly metastatic melanoma cell lines, expression and activity of integrins $\alpha 2\beta 1$ and $\alpha 6\beta 1$ is increased compared with melanocytes and non-metastatic melanoma cells, resulting in enhanced adhesion to the basement membrane components laminin and collagen via these receptors.

Experiments described in chapter 7, show that despite the marked emergence of $\alpha v\beta 3$ in late stages of melanocytic tumor progression, BLM and MV3 melanoma cells can metastasize in the absence of this integrin. The $\alpha v\beta 3$-negative MV3 cell line expresses $\alpha 5\beta 1$, another integrin emerging in melanocytic tumor progression (chapter 4). In chapter 8, the mechanism of binding of these two integrins to their common ligand, fibronectin is investigated. While $\alpha v\beta 3$ binds only to the RGD-sequence in fibronectin, $\alpha 5\beta 1$-binding depends on the PHSRN-synergy site as well. By using various reagents that activate the integrins, it is shown that $\alpha 5\beta 1$ in its high affinity state can bind to RGD in the fibronectin molecule, independent of the synergy site. This implies that $\alpha 5\beta 1$ can be a target integrin for the reported inhibition of melanoma metastasis by RGD-peptides. Therefore, in chapter 9, $\alpha 5\beta 1$-binding antibodies, peptides, and disintegrins (RGD-containing peptides from snake venom) are used to test the hypothesis that $\alpha 5\beta 1$ may be critically involved in metastasis of the $\alpha v\beta 3$-negative MV3 cells. The finding that none of these reagents inhibits metastasis, while each of them blocks $\alpha 5\beta 1$-mediated adhesion to Fn, does not support the hypothesis. In contrast, eristostatin, a disintegrin that does not bind $\alpha 5\beta 1$, is found to block MV3 metastasis. Eristostatin binding to MV3 is RGD-dependent and may involve $\alpha 4\beta 1$. In in vitro adhesion assays, eristostatin does not inhibit adhesion to extracellular matrix components or endothelial cells, suggesting that eristostatin does not act by interference with the initial adhesion of MV3 cells to the vessel wall or to components in the lung tissue. In chapter 9, it is further demonstrated that expression of $\alpha v\beta 3$ in MV3, greatly reduces its metastatic potential.

Chapters 10-12 deal with CD44 molecules in melanoma. CD44 can be detected in all stages of tumor progression in situ and in most cell lines irrespective of their metastatic capacity, while experimental evidence exists for a role for CD44 in melanoma growth and metastasis. The availability of the CD44 ligand hyaluronate may be critical. The findings in chapter 10, that all melanoma cell lines express CD44, that most of them adhere to hyaluronate, but that only the highly metastatic cell lines have a high level of hyaluronate production, may support that idea. As the pattern of CD44 alternative splicing has been related to tumor progression in several malignancies, chapter 11 deals with the expression of CD44 splice variants in human cutaneous melanoma. Gain of CD44v5 and loss of CD44v10 are found to correlate with melanocytic tumor progression in situ, and gain of CD44v5 is also found to be related to the metastatic potential of melanoma cell lines. Finally, in chapter 12, CD44 is shown to be abundantly expressed in uveal melanoma, and the pattern of CD44 alternative splicing in uveal melanoma is shown to be similar to that observed for cutaneous melanoma.

Samenvatting

Uitzaaiing van tumorcellen is de belangrijkste doodsoorzaak bij kankerpatienten. Om de verschillende stappen van tumorprogressie te doorlopen en uit te zaaien, moeten kankercellen in staat zijn om basaalmembranen te passeren en weefsels te invaderen. Hiervoor is het hechtingsgedrag van de cellen van groot belang. Hechting van cellen aan andere cellen en aan de extracellulaire matrix verloopt via cel-adhesie receptoren. Deze zijn onder te verdelen in verscheidene families, te weten de cadherines, selectines, leden van de immunoglobuline superfamilie, integrines en CD44 moleculen. Het feit dat sommige cel-adhesie receptoren signalen genereren die de celproliferatie en differentiatie reguleren, wijst nog verder op hun betrokkenheid bij tumorprogressie en uitzaaiing. Algemene aspecten van de cel-adhesie receptoren en aanwijzingen voor een rol voor deze moleculen in tumorprogressie en uitzaaiing worden behandeld in hoofdstuk 1.

In dit proefschrift worden cel-adhesie receptoren onderzocht in het humane melanoom, een zeer agressieve tumor welke ontstaat uit de pigment-producerende cellen, melanocyten, die zich (doorgaans) in de huid of in het oog bevinden. Om veranderingen in expressie van deze moleculen in relatie tot melanocytaire tumorprogressie te onderzoeken, is gebruik gemaakt van patiënten lesies, te weten benigne nevi, primaire melanomen en uitzaaiingen (de expressie op normale melanocyten in de huid is moeilijk te interpreteren vanwege het nauwe contact met andere huidcellen (keratinocyten)). Verder is de expressie en functie van cel-adhesie receptoren onderzocht in gekweekte normale melanocyten en humane melanoomcellijnen met verschillende capaciteit om uit te zaaien in naakte (thymusloze) muizen.

In hoofdstuk 2 wordt het hechtingsprofiel van gekweekte normale melanocyten bestudeerd. Gevonden wordt dat het expressie-niveau van bepaalde integrines in prolifererende melanocyten hoger is dan in gedifferentieerde melanocyten terwijl andere juist verminderd voorkomen. Dit resulteert voor prolifererende melanocyten in een verlies van het vermogen om aan componenten van de basaalmembraan te hechten. De expressie van E-cadherine op melanocyten en binding aan keratinocyten via deze receptor, wordt gevonden onder beide omstandigheden.

Aangezien E-cadherine sterk tot expressie komt op keratinocyten maar afwezig is op invasieve carcinoomcellen, wordt in hoofdstuk 3 de hypothese onderzocht dat verlies van E-cadherine zich eveneens voordoet tijdens melanocytaire tumorprogressie. De E-cadherine expressie die op gekweekte melanocyten en nevus cellen wordt gedetecteerd, is inderdaad afwezig op frequent uitzaaiiende melanoomcellijnen maar in nevocellulaire nevi in situ wordt ook geen E-cadherine gevonden. Verder wordt expressie gevonden in enkele vergevorderde primaire melanomen en uitzaaiingen. Dus, E-cadherine-gemediëerde adhesie van melanocyten aan keratinocyten is mogelijk een kweek-artefact en verlies van E-cadherine lijkt geen rol te spelen bij melanocytaire tumorprogressie.

In hoofdstuk 4 wordt integrine expressie bestudeerd in lesies van verschillende stadia

van tumorprogressie van het huidmelanoom. Inductie van $\alpha 5\beta 1$ and $\alpha v\beta 3$ expressie in gevorderde stadia zijn de belangrijkste bevindingen. Aangezien het oogmelanoom zowel klinisch als biologisch verschilt van het huidmelanoom zou de expressie van cel-adhesie receptoren voor beide vormen kunnen verschillen. Inderdaad wordt in hoofdstuk 5 beschreven dat alle bestudeerde primaire oogmelanomen $\alpha 5\beta 1$ tot expressie brengen terwijl ze geen $\alpha v\beta 3$ hebben.

Hechting aan de endotheliale basaalmembraan van bloedvaten is een kritische stap in uitzaaiing van kankercellen. Dit idee wordt ondersteund door de bevinding in hoofdstuk 6, dat in frequent uitzaaiende melanoomcellijnen de expressie en activiteit van integrines $\alpha 2\beta 1$ en $\alpha 6\beta 1$ is verhoogd in vergelijking met niet- of sporadisch metastaserende melanoomcellijnen. Dit resulteert in een verhoogd niveau van hechting aan de basaaalmembraancomponenten laminine en collageen via deze receptoren.

De bevindingen beschreven in hoofdstuk 7 laten zien dat, ondanks de opmerkelijke opkomst van $\alpha v\beta 3$ in late stadia van melanocytaire tumorprogressie, BLM en MV3 melanoomcellen in naakte muizen kunnen uitzaaien in afwezigheid van dit integrine. De $\alpha v\beta 3$-negatieve cellijn MV3, brengt een ander integrine dat opkomt in late stadia van tumorprogressie tot expressie, namelijk $\alpha 5\beta 1$. In hoofdstuk 8 wordt het mechanisme van hechting van beide receptoren aan hun gemeenschappelijke ligand, fibronectine onderzocht. Terwijl $\alpha v\beta 3$ alleen de RGD-sequentie in het fibronectine molecule bindt, is binding van $\alpha 5\beta 1$, zowel van RGD als van de synergistische regio met de PHSRN-sequentie afhankelijk. Met verscheidene reagentia die de affiniteit van integrines voor hun ligand verhogen, wordt echter gedemonstreerd dat $\alpha 5\beta 1$ in de actieve (hoog-affine) toestand, RGD in fibronectine kan binden onafhankelijk van de PHSRN-sequentie. Dit impliceert dat $\alpha 5\beta 1$ een doelwit-integrine kan zijn voor de eerder beschreven remming van melanoom uitzaaiing met RGD-peptiden. Daarom worden in hoofdstuk 9 $\alpha 5\beta 1$-bindende antilichamen, peptiden en disintegrines (RGD-bevattende peptiden geïsoleerd uit slangegif) gebruikt, om de hypothese te testen dat $\alpha 5\beta 1$ van essentiëel belang is voor uitzaaiing van de $\alpha v\beta 3$-negatieve cellijn MV3. De bevinding dat geen van deze reagentia uitzaaiing van MV3 cellen remt terwijl ze allemaal de hechting aan fibronectine via $\alpha 5\beta 1$ blokkeren, bevestigt deze hypothese niet. Een disintegrine met een andere bindings-specificiteit, eristostatine, blokkeert de uitzaaiing van MV3 zeer efficiënt. Eristostatine bindt aan MV3 cellen en deze binding is RGD-afhankelijk en verloopt mogelijk via $\alpha 4\beta 1$, maar met in vitro adhesie testen wordt geen remming gevonden van hechting aan extracellulaire matrix componenten of aan endotheelcellen. Dit suggereert dat het effect van eristostatine niet te wijten is aan verhindering van de initiële hechting aan de vaatwand of componenten uit het longweefsel. In hoofdstuk 9 wordt verder aangetoond, dat expressie van $\alpha v\beta 3$ in MV3 de capaciteit tot uitzaaien drastisch reduceert.

De hoofdstukken 10-12 behandelen de expressie van CD44 in het humane melanoom. CD44 kan worden gedetecteerd in alle stadia van melanocytaire tumorprogressie in situ en in de meeste melanoomcellijnen onafhankelijk van hun vermogen tot uitzaaien. Anderzijds

hebben experimentele studies aangetoond dat CD44 een rol kan spelen in groei en uitzaaiing van melanoomcellen. De beschikbaarheid van het ligand voor CD44, hyaluronzuur, zou van kritisch belang kunnen zijn. De bevinding in hoofdstuk 10 dat alle melanoomcellijnen CD44 tot expressie brengen en dat de meeste van hen aan hyaluronzuur hechten maar dat alleen de frequent uitzaaiende melanoomcellijnen een hoge productie van hyaluronzuur hebben, lijkt dit idee te bevestigen. Aangezien het patroon van alternatieve splicing van CD44 gerelateerd is aan tumorprogressie in een aantal verschillende vormen van kanker, behandelt hoofdstuk 11 de expressie van CD44 splice varianten in het humane huidmelanoom. Expressie van CD44 moleculen die het v5-exonproduct bevatten, blijkt te correleren met melanocytaire tumorprogressie in situ en met de capaciteit tot uitzaaien van melanoomcellijnen. Andersom, blijkt verlies van CD44 moleculen met het v10-exonproduct gerelateerd te zijn aan melanocytaire tumorprogressie in situ. Ten slotte laat hoofdstuk 12 zien dat CD44 ook in oogmelanomen sterk tot expressie komt en dat het patroon van alternatieve splicing van CD44 in oogmelanomen overeenkomt met dat in huidmelanomen.

Ook hier mag de constatering, dat een proefschrift het werk van velen omvat, niet ontbreken. Op deze plaats wil ik graag diegenen bedanken die met raad of daad hielpen bij het in dit boekje beschreven werk. Enkelen wil ik met naam noemen.

Allereerst de collega's van Pathologie, bedankt voor jullie behulpzaamheid en de gezellige sfeer. Dirk Ruiter en Goos van Muijen, jullie wil ik bedanken voor het in mij gestelde vertrouwen. De ruime mate van vrijheid die ik kreeg om het project vorm te geven, gecombineerd met het altijd snelle en kritische commentaar op mijn schrijfsels, maakte het erg plezierig om onder jullie hoede mijn eerste onderzoeksbaan te doorlopen. Kees Jansen en Annemieke van Kraats, van immuunhistochemie tot flowcytometrie en van adhesie assay tot transfectie assay, ik kon steeds een beroep op jullie doen. Zonder die hulp zouden de omvang en het niveau van dit werk een stuk minder zijn. Ine Cornelissen, al de muizeproeven deden we samen met jou. Door jouw ervaring werden ook de groots opgezette proeven, soepel en snel afgewerkt.

Studenten Paul ten Berge, Debbie Nicastia en Leo Smeets, het was ontzettend leuk om met jullie samen te werken. Paul, hopelijk is de enorme hoeveelheid coupes die jij hebt gekleurd tevens een aanzet tot jouw eigen boekje. Debbie en Leo, het spijt me dat ik niet de tijd heb gevonden om verder te gaan met jullie werk, waardoor in dit proefschrift geen hoofdstukken staan over migratie van de vele ECM klonen, of over IAP, calreticuline en pp125FAK.

Carl Figdor, de eerste adhesie-testjes deed ik tijdens de stage op jouw lab. Gelukkig kon ik ook daarna nog af en toe van je kennis van de integrins gebruik maken en klopte ik nooit vergeefs aan voor antilichamen. Dit is essentieel geweest voor een aantal hoofdstukken uit dit proefschrift. Arie Pennings en Piet van Erp wil ik bedanken voor het steeds startklaar houden van de flowcytometers op Hematologie en Dermatologie. Hierdoor werd het meten voor mij simpelweg aanschuiven, buisje eronder en printen maar. De dierenverzorgers van het CDL wil ik bedanken voor al het werk dat komt kijken bij het huisvesten van de nudes.

Eberhard Klein, I very much enjoyed my stay in Ulm and our enthusiastic discussions over the phone. Paolo Mignatti and Roberta Mazzieri, I thank you for the time I could stay in Pavia learning the amnion-invasion assay. I regret that I've not been able to get the assay running good enough to efficiently test changes in invasive behavior. Drs. Stefan Niewiarowski and Kenneth Yamada, it has been very stimulating to collaborate with you.

Tot slot: Papa en mama, bedankt voor een zorgeloze jeugd zonder gehamer op het belang van goede studieresultaten, waardoor ik op een wat later tijdstip zowaar nog plezier heb gevonden in studeren, en Helen, bedankt voor je betrokkenheid en voor je besluit om mee te gaan.

ERIK

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 11 April 1965 te Dieren. Na het doorlopen van de HAVO en het VWO van 1977 tot 1985 aan het Stedelijk Lyceum te Zutphen, werd de militaire dienstplicht vervuld. In 1986 werd de studie Biologie gestart aan de Katholieke Universiteit te Nijmegen. Voor de richting Medische Biologie werden twee hoofdvakken gevolgd: Immunologie bij Prof. C.G. Figdor (afd. Immunologie, het Nederlands Kanker Instituut, Amsterdam) en Moleculaire Neuroendocrinologie bij Prof. G.J.M. Martens (afd. Experimentele Dierkunde, Katholieke Universiteit Nijmegen). Hij slaagde voor het doctoraal examen in mei 1991. In diezelfde maand kreeg hij een aanstelling als wetenschappelijk medewerker op de afdeling Pathologie van het Radboud Ziekenhuis te Nijmegen. Hier werd onder begeleiding van Prof. D.J. Ruiter en Dr. G.N.P. van Muijen, gewerkt aan een door de Nederlandse Kankerbestrijding gefinancierd project, waarvan de resultaten in dit proefschrift zijn beschreven. Vanaf april 1996 zal hij als fellow van de Nederlandse Kankerbestrijding werkzaam zijn bij Dr. Kenneth Yamada, Laboratory of Developmental Biology, National Institutes of Health, Bethesda, MD.

Martens GJM, Piosik PA, Danen EHJ. Evolutionary conservation of the 14-3-3 protein. *Biochem Biophys Res Commun* **184**, 1456-1459, 1992.

Danen EHJ, Van Muijen GNP, Ten Berge PJM, Ruiter DJ. Integrins and melanoma progression. *Recent Res Cancer Res* **128**, 119-132, 1993.

Danen EHJ, Van Muijen GNP, Van de Wiel-van Kemenade E, Jansen KFJ, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. *Int J Cancer* **54**, 315-321, 1993.

Ten Berge PJM, Danen EHJ, Van Muijen GNP, Jager MJ, Ruiter DJ. Integrin expression in uveal melanoma differs from cutaneous melanoma. *Invest Ophthalmol Vis Sci* **34**, 3635-3640, 1993.

Danen EHJ, Ten Berge PJM, Van Muijen GNP, Van 't Hof-Grootenboer AE, Bröcker E-B, Ruiter DJ. Emergence of $\alpha 5\beta 1$ fibronectin- and $\alpha v\beta 3$ vitronectin-receptor expression in melanocytic tumor progression. *Histopathol* **24**, 249-256, 1994.

Van Muijen GNP, De Vries TJ, Danen EHJ, Quax PHA, Verheijen JH, Ruiter DJ. Neue Gesichtspunkte der Melanompathogenese: Eigenschaften metastasierender und nicht-metastasierender humaner Melanomzellen. Page 101-112 In: *Jarbuch der Dermatologie, Tumoren und Haut.* Eds: Macher E, Kolde G, and Bröcker E-B, Biermann Verlag, Germany, 1994.

Van Muijen GNP, Danen EHJ, De Vries TJ, Quax PHA, Verheijen JH, Ruiter DJ. Properties of metastasizing and non-metastasizing human melanoma cells. *Recent Res Cancer Res* **139**, 105-122, 1995.

Van Muijen GNP, Danen EHJ, Veerkamp JH, Ruiter DJ, Lesley J, Van den Heuvel LPWJ. Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacities. *Int J Cancer* **61**, 241-248, 1995.

Danen EHJ, Jansen KFJ, Van Kraats AA, Cornelissen IMHA, Ruiter DJ, Van Muijen GNP. Alpha-v integrins in human melanoma: gain of $\alpha v\beta 3$ and loss of $\alpha v\beta 5$ are related to tumor progression in situ but not to metastatic capacity of cell lines in nude mice. *Int J Cancer* **61**, 491-496, 1995.

De Waard I, Blom J, Griffioen P, Schrier P, Hoogendoorn K, Beverstock N, Danen EHJ, Jager MJ. Establishment and characterization of a uveal melanoma cell line. *Int J Cancer* **62**, 155-166, 1995.

Manten-Horst E, Danen EHJ, Smit L, Snoek M, Le Poole C, Van Muijen GNP, Pals ST, Ruiter DJ. Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential. *Int J Cancer* **64**, 182-188, 1995.

Danen EHJ, Van Muijen GNP, Ruiter DJ. The role of integrins as signal transducing adhesion molecules in human cutaneous melanoma. *Cancer Surveys* **24**, 43-67, 1995.

Danen EHJ, Ruiter DJ, Van Muijen GNP. Mechanisms of melanoma cell adhesion to fibronectin. *Biochem Soc Transactions* **23**, 403S, 1995.

Creyghton W, De Waard-Siebinga I, Danen EHJ, Jager, MJ. Cytokine-mediated modulation of integrins, ICAM-1, and CD44 expression on human uveal melanoma cells in vitro. *Melanoma Res* **5**, 235-242, 1995.

Danen EHJ, Aota CI, Van Kraats AA, Yamada KM, Ruiter DJ, Van Muijen GNP. Requirement for the PHSRN synergy site for cell adhesion to the central cell binding domain in fibronectin depends on the activation state of integrin $\alpha 5\beta 1$. *J Biol Chem* **270**, 21612-21618, 1995.

Bakker ABH, Marland G, De Boer AJ, Huijbens RJF, Danen EHJ, Adema GJ, Figdor CG. Generation of anti-melanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro. *Cancer Res* **55**, 5330-5334, 1995.

Danen EHJ, Jansen KFJ, Klein CE, Smit NPM, Ruiter DJ, Van Muijen GNP. Loss of adhesion to basement membrane components but not to keratinocytes in proliferating melanocytes. *Eur J Cell Biol*, **in press**, 1996.

Danen EHJ, Ten Berge PJM, Van Muijen GNP, Jager MJ, Ruiter DJ. Expression of CD44 and the pattern of CD44 alternative splicing in uveal melanoma. *Melanoma Res*, **in press**, 1996.

Danen EHJ, De Vries TJ, Morandini R, Ghanem GG, Ruiter DJ, Van Muijen GNP. E-cadherin expression in human melanoma. *Melanoma Res*, **in press**, 1996.

Wesseling P, Danen EHJ, Link M, van Muijen GNP, Ruiter DJ. Mismatch between microvascular integrins and extracellular matrix in glioblastoma multiforme: a potential cause of glomeruloid microvascular proliferation. *Submitted.*